STUDIES ON STATIONARY PHASE *VIBRIO* sp. 2

BY

NICHOLAS GEORGE CAR

Submitted in partial fulfilment of the requirements for the degree of Ph.D. in the Faculty of Science, University of Cape Town.

March, 1987

Acknowledgments

I would like to thank my supervisor, Professor David R. Woods for all his encouragement and help in many ways throughout my postgraduate studies.

I wish to thank my wife, Catherine, and my children for allowing me the valuable time from fatherly duties to enable me to pursue postgraduate studies.

Many thanks are also due to Mrs. Barbara Young for her assistance in the electron micrography studies.

I acknowledge a CSIR Research Assistantship for support of this work.

ABBREVIATIONS

[^{14}C-me]globin	[^{14}C]-methyl apohaemoglobin
2-ME	2 mercaptoethanol
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
c.p.m.	counts per minute
cml	chloramphenicol
d	day(s)
dATP	deoxyadenosine 5'-triphosphate
DEAE cellulose	dietylaminoethyl cellulose
EDTA	etylenediaminetetraacetic acid
GTP	guanosine 5'-triphosphate
h	hour(s)
m	minute(s)
o.p.m.	orbitals per minute
PEI-cellulose	polyethyleneimine cellulose
pHMB	p- hydroxymercuribenzoate
PMSF	phenylmethylsulphonyl fluoride
ppApp	adenosine 5'-diphosphate, 3'-diphosphate
ppGp	guanosine 5'-diphosphate, 3'-phosphate
ppGpp	guanosine 5'-diphosphate, 3'-diphosphate
pppApp	adenosine 5'-triphosphate, 3'-diphosphate
pppGpp	guanosine 5'-triphosphate, 3'-diphosphate
TCA	trichloroacetic acid

CONTENTS

SUMMARY

Vibrio sp. 2 stationary phase cells are novel and interesting in that they are able to support phage growth in standing cultures, but not in shaken (aerated) cultures. Many physiological and morphological characteristics change when Vibrio sp. 2 stationary phase cells are removed from aeration: the relatively high levels of protein synthesis (Robb et al., 1977; 1978) decrease, with a concomitant increase in the levels of RNA synthesis; protein degradation rises from 1 $\%h^{-1}$ to 2,9 $\%h^{-1}$, and whilst the average cell length decreases, the range of cell lengths markedly increases.

The magic spot nucleotides, ppGpp and pppGpp, which are present in stressed exponential phase Vibrio sp. 2 cells, are not detectable in stationary phase Vibrio cells.

The specific proteolytic activity of shaking stationary phase cell-free extracts against the foreign protein [^{14}C-me]globin was slightly higher than that of extracts from standing or exponential phase cells, while the specific proteolytic activity against [^{125}I]-insulin was slightly lower. On the basis of inhibitor studies and subcellular distribution, the proteolytic activities of the three types of extract differed.

The addition of exogenous ATP to cell-free extracts either stimulated (Car & Woods, 1984) or depressed proteolytic activity depending on the procedure used to prepare the extracts. The proteolytic activity of

fractions containing substantial amounts of membrane material, from all three types of extract, were markedly depressed by ATP. On preincubation of cell-free extracts from exponentially growing cells prior to assay of proteolytic activity, the activity was markedly stimulated (two- to four-fold). The stimulation, however, varied greatly between independently produced extracts.

ATP had a much smaller stimulatory effect on preparations free of cell wall material from both types of stationary phase cells (the stimulation was less than two-fold), and the stimulation was not affected by preincubation of the extracts. Extracts prepared from starving cells, previously in exponential growth, were affected by the addition of ATP in a similar manner to that observed with stationary phase extracts (Car & Woods, 1984).

Exponential and both types of stationary phase Vibrio sp. 2 cells have ATP-stimulated and ATP-depressed activities separable by ion-exchange chromatography, in addition to several other proteolytic activities. All types of Vibrio sp. 2 cells have a similar complement of proteolytic activities.

CHAPTER ONE

INTRODUCTION.

1.1. Isolation of Vibrio species.

Investigation of the causes of leather decay during tanning processes resulted in the isolation of several Gram-negative halotolerant bacteria (Thomson et al., 1972), which due to their proteolytic and collagenolytic activity have been implicated in the decay process (Woods et al., 1972). Several isolates were classified as Achromobacter species and investigated because of their aerobic collagenolytic activity (Welton & Woods, 1973; 1974), and protease production (Keil-Dlouha et al., 1976). During this work, a highly collagenolytic strain was identified as Achromobacter iophagus (Welton & Woods, 1973). A co-isolated strain was identified as an Achromobacter, but of undetermined species and designated Achromobacter sp. 2. A. iophagus has subsequently been reclassified as Vibrio alginolyticus (Reid et al., 1980) and as Achromobacter sp. 2 is closely related to V. alginolyticus, it is felt that Achromobacter sp. 2 is better designated as Vibrio sp. 2, although its precise relationship to V. alginolyiticus has not been established.

Genetic investigation of V. alginolyticus has been hampered by the lack of a phage for the strain and a transformation system. However, phages for Vibrio sp. 2 were isolated from a hide soak solution, or induced by U.V. irradiation. One such phage, Alpha 3a, was isolated as a

spontaneous plaque on a lawn of Vibrio sp. 2 (Thomson, 1973) and several investigations of the biology of Vibrio sp. 2 and Alpha 3a have been undertaken (Thomson, 1973; Woods, 1976; Robb et al., 1977; 1978; 1980; 1982). Although it was not possible to transform Vibrio sp. 2 with bacterial DNA (Thomson 1973), it was possible to transfect spheroplasts of Vibrio sp. 2 with phage Alpha 3a DNA (Thomson & Woods, 1973). Mitomycin C and nitrosoguanidine have been found to induce the phage (Robb, 1979), and evidence for a cryptic prophage in Vibrio has been reported (Thomson & Woods, 1974). Phage Alpha 3a was shown to mediate unstable generalised transduction (Woods & Thomson, 1975).

During this work on transduction, one of the transductant clones (Strain 14) was found to support Alpha 3a growth only in stationary phase (Woods, 1976), and phage growth was found to be sensitive to aeration, that is phage growth occurred in non-aerated (standing) stationary phase cultures, but not in aerated (shaken) stationary phase cells.

1.1.1. Stationary phase phage growth.

Phage Alpha 3a replication in non-growing cells was studied in detail by Robb (1979) and Robb et al. (1977; 1978; 1980). Their results are summarised below. Both Vibrio sp. 2 and strain 14 support phage growth in stationary phase, but unlike Vibrio sp. 2, strain 14 did not allow phage propagation in exponential phase. Alpha 3a growth in stationary phase shows a long and variable latent period of about 7 h and a 4- to

5-fold increase in burst size compared to that found in exponential phase. Stationary phase phage growth is very sensitive to culture conditions, and growth only occurs at oxygen concentrations of about 5 p.p.m. Standing stationary phase cells can support phage growth for at least 16 days, and show increased viability, RNA synthesis, membrane transport, intracellular ATP levels and U.V. resistance compared to shaken stationary phase cells, but the rate of protein synthesis is much higher in shaken than in standing stationary phase cells.

It is generally accepted that bacteriophage replication is inhibited in stationary phase bacteria (Adams, 1959; Hayes, 1968). However, variations in physiological processes have been defined for spore forming bacteria, such that phage Ø infected Bacillus subtilis cells committed to sporulation will not support phage growth until after spore germination (Yehle & Doi, 1967; Sonnenshein & Roscoe, 1969; Ito et al., 1973).

Stationary phase phage growth has been investigated (Woods, 1976) and it is likely that this phenomenon is a property of the Alpha 3a - Vibrio sp. 2 system rather than a property of either the phage or bacterium alone.

Robb (1979) has proposed a phase variation phenomenon analogous to the imm^{+}/ imm^{-} variation in lambda infected Escherichia coli (Lewin, 1977) to account for the variation in phage susceptibility. She suggested that fluctuations in phage repressor levels (from the cryptic phage carried by Vibrio sp. 2) prevent superinfection in aerated stationary phase cells, but that transcriptional changes allow phage growth in standing stationary phase (Robb et al., 1977). Biochemical

differentiation by transcription regulation has been suggested (Robb, 1979) to account for stationary phase phenomena, and is supported by a rifampicin mutant of Vibrio 1p (which only supports phage growth in exponential phase) that is able to support stationary phase phage growth.

Phage Alpha 3a receptors on Vibrio sp. 2 have been shown to be associated with the leucine binding protein, as mutants which have lost the leucine uptake system were completely resistant to phage infection, and could not absorb Alpha 3a (Robb, 1979; Robb et al., 1982).

1.1.2. Naming of Vibrio strains.

Some confusion exists about the naming of the Vibrio sp. 2 strains (formerly Achromobacter sp. 2). Transductant strains 14 and 15 were only able to support Alpha 3a growth in stationary phase, and could not mediate transduction (Robb, 1979). An "Achromobacter sp. 2" also referred to as "Achromobacter w.t." by Woods (1976) and Robb et al., (1977; 1978), was unable to support stationary phase phage growth. That particular exceptional strain has been termed Vibrio 1p (for "log phase" development, Robb 1979; Ch. 6). The wild type strain, Vibrio sp. 2, called Achromobacter sp. 2 by Robb (1979), grows on unsupplemented minimal medium and supports Alpha 3a growth in both exponential and standing stationary phase. This was the only strain used throughout the work reported in this thesis, and the characteristic of exponential and stationary phase phage growth was regularly tested, with each subculturing.

1.2. Prolonged bacterial survival.

A culture of bacteria inoculated into fresh medium typically shows several phases of growth; lag phase, exponential phase, stationary phase and the decline phase, although these phases may be subdivided to give seven phases in all (Koch, 1971). This sequence is not inevitable and medium composition influences various phases. For example, E. coli does not have a lag phase when grown in media containing limiting glucose (Cohen & Arbogast, 1950). In a complex medium, the exponential phase is terminated gradually when the cells begin to exhaust the available nutrients, when toxic substances accumulate or the pH becomes unfavourable for growth (Dean & Henshelwood, 1966). The length of the stationary phase and loss of viability in the decline phase is influenced by many factors, ,including the composition of the growth medium. Thus Ramagopal (1984) showed that E. coli lost viability more rapidly in a complex medium (Luria-Bertani broth) than in a phosphate buffered glucose-yeast extract medium.

Survival of bacterial cells under chronic starvation has been studied, particularly for soil and marine bacteria, which would in situ face extended periods of starvation. Viability loss during starvation is influenced by many factors including medium composition and ambient conditions. The survival of Streptococcus lactis during starvation was markedly enhanced by Mg^{2+} and low temperatures (Thomas & Batt, 1968) whereas agitation and aeration tended to decrease survival. The presence of amino acids in the medium enhanced survival of the cells, in

the presence of magnesium ions, whereas the presence of fermentable carbohydrates accelerated death of the organisms.

The presence of endogenous energy and carbon reserves, such as poly-beta-hydroxybutyrate or glycogen serve to enhance the viability of the soil organisms Sphaerotilus discophorous (Stokes & Parson, 1968) and Aerobacter aerogenes (Strange et al., 1961) during starvation. Boylen & Ensign (1970) have reviewed the half-lives reported for various bacteria during starvation and found that most have half-lives of less than 5 days, with the half-life starvation time of Arthrobacter crystallopoietes being the longest at 100 days.

Bacteria in the open ocean, an oligotrophic environment, show remarkably long survival times, measured in years (Morita, 1982). Ant-300, a psychrophilic marine Vibrio strain isolated from the Antarctic convergence, has been extensively studied by Morita and coworkers. On starvation, Ant-300 cells decreased in size changing from rod shaped to coccoid, and after 3 weeks of starvation the cells were able to pass through a 0,4 um filter (Novitsky & Morita, 1976). These small cells had a normal cell structure, as seen by electron microscopy, except for an enlarged periplasmic space. The decrease in cell size was accompanied by a 2- to 9-fold increase in the number of viable cells (Novitsky & Morita, 1977) and a decrease in the number of nuclear bodies. Endogenous respiration in these circumstances fell rapidly to a low level (< 0,01 %) within 7 days and remained constant thereafter. The level of protein and DNA per cell initially decreased then increased again to between 2- and 3-fold more than the minimum level (Amy et al., 1983 a). These changes occurred during the first six weeks of

starvation, so that the cells had depleted much of their stored products and had become ultramicrocells, after which a stable pattern for long-term starvation was seen.

At low nutrient levels, continued cell division in the absence of growth results in ultramicrocells (Henrici, 1928). Ultramicrocells have been reported in both soil and marine samples (review, Morita, 1982). Miniaturisation increases the cells' surface/volume ratio, and may increase the cells' ability to scavenge energy-yielding substrates. However, Baker & Park (1975), found that although Vibrio sp. NCTC 4716, which occurred as rods in exponential phase, changed to spheres in the decline phase, the spheres were not viable and the survival of the culture depended on the few rod forms that remained.

The initial increase in the number of viable cells upon starvation has been interpreted as a strategy for starvation survival (Novitsky & Morita, 1978 a). Populations of Ant-300 suspended at low cell densities showed greater increases in cell numbers than populations suspended at higher densities, as well as increased longevity as the number of viable cells declined, so that after 70 weeks over 15 times the original number were still viable. Starvation of Ant-300 prior to exposure to increased hydrostatic pressure also enhanced survival (Novitsky & Morita, 1978 b).

The increase in RNA content observed in Ant-300 may be an adaptive response to starvation, as seen in slowly growing E. coli cells which have "extra" RNA (Kock, 1971). Upon alleviating starvation, Ant-300 recovered and showed the usual bacterial growth curve (Amy et al., 1983 b). The length of the lag phase during recovery was found to be

/

directly proportional to the length of prior starvation. Thus the cells appeared to pass more deeply into dormancy with starvation time.

The pattern of starvation-survival reported for Ant-300 is one of three patterns reported by Amy & Morita (1983 c) for many freshly isolated marine bacteria. The patterns showed either an initial increase or decrease in viable cells, to a constant number. In each case, the cells retained the ability to actively metabolise added nutrients. The cells changed their protein patterns on starvation (Amy & Morita, 1983 c) and showed chemotaxis, a response not seen with freshly harvested cells (Geesey & Morita, 1979). Morita (1982) proposed that these metabolic and morphological changes occur to enable the cell to survive starvation, at the same time preparing it to benefit maximally from any increase in available nutrients.

Although starvation survival in mesophiles such as E. coli has not been as extensively studied as the psychrophiles mentioned above, protein degradation has been shown to play a role in the survival of starved E. coli and Salmonella typhimurium (1.5.1) and a set of unique proteins has been shown to be synthesised at the beginning of carbon starvation in E. coli (Groat & Matin, 1986). Some of these proteins are heat shock proteins.

1.3. Stringent control in bacteria.

In bacterial cells, the rate of protein synthesis varies with the growth rate. However, the rate of amino acid incorporation per ribosome is

constant over a wide range of growth rates (Maaloe & Kjeldgaard, 1966). The protein synthesis rate is controlled by the number of ribosomes per cell, or more precisely, by the rate at which ribosomes are produced. The exponential phase of bacterial growth is referred to as "balanced" because the production of cell components is perfectly coordinated (Maaloe & Kjeldgaard, 1966).

When the growth of a bacterial cell is perturbed, for example by amino acid starvation, the cell responds by making a rapid, complex and varied set of adjustments to its metabolism. This adjustment is termed the stringent response, consisting of the restriction of transcription of ribosomal ribonucleic acid (rRNA) and transfer-RNA (t-RNA), carbohydrate, phospholipid, lipid, de novo nucleotide synthesis, transport of exogenous nucleobases and glycosides, and an increase in the rate of protein degradation (reviews, Cashel, 1975; Gallant & Lazzarini, 1976; Gallant, 1979).

The hallmark of the stringent response is the drastic (about 3-fold) reduction in the rate of total RNA accumulation (Sands & Roberts, 1952). Stable RNA, that is rRNA and tRNA, synthesis rates fall 10- to 20-fold. The effect on mRNA is varied, the synthesis of trp and phage Ø 80 mRNA is unaffected, that of ribosomal protein and some enzymes (for example, ornithine transcarbamylase) is restricted, while the synthesis of his and trp messengers is stimulated (review, Gallant, 1979). O'Farrell (1978) used two-dimensional gel electrophoresis to show that of the over 300 E. coli proteins resolved by the procedure, the production of 25% was stimulated and the production of a further 25% was inhibited during stringency.

Stringent control therefore entails a major re-adjustment of metabolic as well as transcriptional patterns. As the major portion of the available energy in an exponentially growing bacterium is required for the synthesis of ribosomes, the cessation or curtailment of ribosome synthesis is an important mode of energy conservation.

Stringent control is abolished by a single site mutation, conferring the "relaxed control" phenotype on the cell (Stent & Brenner, 1961). The locus, initially called "RC" (for relaxed control), is now termed relA.

Cashel & Gallant (1969) found two unusual guanine nucleotides, since identified as guanosine 5'-diphosphate, 3'-diphosphate (ppGpp) and guanosine 5'-triphosphate, 3'-diphosphate (pppGpp), which accumulated within seconds of triggering the stringent response in relA$^+$ cells, but not in relA$^-$ cells.

1.3.1. Other highly phosphorylated nucleotides.

Apart from the two nucleotides mentioned, many other highly phosphorylated nucleotides have been found in bacteria. In E. coli itself, an analogue of GTP, ppGp, has been found (Gallant et al., 1976) which decreases rapidly in concentration upon energy source downshift, and the authors suggest it may be a positive effector of cellular processes, including RNA accumulation.

The presence of ppGpp and pppGpp has been demonstrated in a wide variety of prokaryotes, including Gram-positive bacteria (review, Silverman &

Atherley, 1979). The presence of several highly phosphorylated adenine nucleotides has been shown in B. subtilis (Rhaese et al., 1976; 1977; 1978). The guanine nucleotides ppGpp and pppGpp are synthesised by B. subtilis cells during vegetative growth, whereas the adenine analogues ppApp and pppApp are synthesised during sporulation, indicating a further role for highly phosphorylated nucleotides in gene expression.

Actinomycetes produce an enzyme capable of synthesising purine nucleotide 3' diphosphates during growth, but the function of these nucleotides remains obscure (Silverman & Atherley, 1979).

Although ppGpp appears to be ubiquitous (Silverman & Atherley, 1979) it has not been established if it has the same function in other bacteria as it has in E. coli.

1.3.2. ppGpp and pppGpp metabolism.

The synthesis of these nucleotides occurs on the ribosome, by the relA gene product (stringent factor) which is removable from ribosomes in a high salt wash. This factor catalyses the transfer of the γ -pyrophosphoryl group of ATP to the 3' hydroxyl of GDP or GTP to yield ppGpp or pppGpp respectively. In vitro ppGpp and pppGpp synthesis has been demonstrated (Haseltine et al., 1972), and it has also been shown that, dissociated from the ribosome, the purified stringent factor has a low level of pyrophosphotransferase activity (Sy et al., 1973).

The synthesis of these nucleotides is stimulated by the codon-specific binding of uncharged tRNA to the ribosome A site (review, Block & Haseltine, 1974). The major pathway of synthesis seems to be:

$$GTP \xrightarrow{1} pppGpp \xrightarrow{2} ppGpp \xrightarrow{3} GDP$$

Several enzymes appear to be able to catalyse step 2. Step 1 is catalysed by the relA gene product as discussed, and step 3 is catalysed by the spoT gene product (Fiil et al ., 1977).

The concentration of ppGpp in vitro has been inversely correlated with the rate of stable RNA synthesis, under a great variety of conditions including carbon source or energy limitation, temperature increase, and amino acyl-tRNA limitation (review, Cashel, 1975; Gallant & Lazzarini, 1976). However, there have been reports showing that this relationship did not always hold since wild type E. coli can show the stringent response without the accumulation of ppGpp (review, Gallant, 1979; Spadaro et al., 1981). For this reason, a causal role for ppGpp in stringency has been questioned.

There is a large body of literature reporting effects of ppGpp on in vitro transcription (review, Gallant, 1979). Many of these reports show preferential inhibition of rRNA in in vitro transcription systems, while others show that there are no specific effects. The controversy probably reflects the critical sensitivity of the in vitro experimentation system to assay conditions (Gallant, 1979). Several models have been proposed for the mechanism of rRNA synthesis control (review, Nomura et al., 1985).

The passive control model, developed along the lines of Maaloe's model for r-protein synthesis (Maaloe, 1979), assumes that the total rate of RNA transcription is limited by the number of RNA polymerase molecules, that rRNA promoters are constitutive, and that competition between these and other regulated non-ribosomal promoters alters the activity of the rRNA promoters. However, it has been shown that RNA polymerase molecules are present in excess in the cell (Gausing, 1980), and in gene dosage experiments, the transcriptional activity of rRNA operons can be changed without apparent changes in competing non-ribosomal transcriptional activities (review, Nomura et al., 1985).

Bremer and coworkers (Ryals et al., 1982 a; 1982 b) have shown that the reduction in specific stable RNA gene activity (the rate of stable RNA synthesis relative to the total instantaneous rate of RNA synthesis) is strongly correlated with the rise in ppGpp accumulation, and propose that ppGpp is the sole effector involved in stable RNA synthesis regulation.

They propose a model along the lines proposed by Travers (review, Lamond & Travers, 1985) that there are two forms of RNA polymerase, one (through the mediation of ppGpp) has a reduced affinity for its binding sites, so that those promoters for which polymerase availability is limiting would no longer support efficient initiation. Little et al. (1983) have isolated a RNA polymerase mutant (rpoB) in which the relationship between ppGpp and stable RNA gene activity has been altered, in that the RNA polymerase is about 20-fold more sensitive to ppGpp than the polymerase in an otherwise isogenic strain. Studies of

the promotor regions of stable RNA genes have shown additional features to those shown by other promoters in E. coli (review, Lamond, 1985) in that they have a stimulating element upstream of the -35 region and a discriminator between the -10 region (TATAAT) and the transcription start site. Removal of the stimulating region (at least for the $tRNA^{tyr}$ gene) causes a 10- to 12-fold decrease in promotor activity, and the GC-rich discriminator is an important determinant of stringent control.

The effect of growth rate control is the balanced production of the individual components of the translational apparatus (review, Nomura et al., 1985), such that they are always produced in equimolar ratios, and their relative production increases with the cells' growth rate. A model proposed by Nomura et al. (1985) for growth rate control, envisages a feedback mechanism where free, functional but non-functioning ribosomes inhibit the transcription of stable RNA genes.

This model is based on gene dosage experiments, in which the number of rRNA operons in a cell is increased by introducing plasmid-encoded rRNA operons. The total amount of rRNA synthesis does not change, as the level of expression of individual operons is decreased. The effect is not produced if the extra copies of rRNA operons do not produce a functional rRNA transcript (Jinks-Robertson et al., 1983). However, the model does not distinguish between a direct effect of the free non-translating ribosomes per se, or an indirect effect of a trans-acting ribosomal product, which might easily be ppGpp.

1.4. Protein synthesis during starvation.

Within a few minutes of amino acid starvation, the protein synthesis rate drops to less than 10% of its value prior to starvation (Brunschede & Bremer, 1971) and low molecular mass proteins are synthesised preferentially, the molecular mass distribution being consistent with premature release of incomplete peptides from ribosomes.

Residual protein synthesis proceeds at about the same rate in stringent and relaxed cells (review, Gallant, 1979), but relaxed cells show deficient protein synthesis, for example, beta-galactosidase is produced in *relA*$^-$ cells at a lower rate than in *relA*$^+$ cells, and also the enzyme activity per unit monomer is reduced by a factor of three (Hall & Gallant, 1972). The defect comprises both a transcriptional component and a translational component (Foley *et al.*, 1981). The relaxed cells may substitute an amino acid whose codon is similar to that of the deficient amino acid. Edelman & Gallant (1977) showed that during starvation, *relA* mutants incorporated [^{35}S]-cysteine for arginine in flagellin (arginine has a single base coding difference from cysteine). Flagellin does not normally have this amino acid. Furthermore, O'Farrell (1978), using two dimensional gel analysis of *E. coli* proteins found that cells starved of some amino acids had proteins which showed electrophoretic heterogeneity in the iso-electric focusing dimension, producing satellite spots related to the normal spots by integral charge changes, as would be seen by substitution of amino acids with different charge from the normal amino acid.

The mechanism by which mistranslation, which is seen in *relA*$^-$ cells during starvation, is suppressed in *relA*$^+$ starving cells is not known. Gallant (1979) has developed the 'kinetic amplification' idea put forward by Ninio (1975), which envisages that if a correct and incorrect substrate can both bind an enzymatic site with identical rates of association, but the incorrect substrate dissociates from the complex more rapidly, and this is followed by an irreversible step, any slowing down of the reaction would favour the correct substrate. The presence of ppGpp might cause such a slowing down.

There are, however, conflicting data which show that neither the rate of GTP hydrolysis, which signals the selection of the ternary complex, nor the rate of peptide formation, which signals the acceptance of the aa-tRNA, is affected by ppGpp (Dix *et al.*, 1983). This would seem to preclude a direct effect by ppGpp on the rate constants for substrate selection by the ribosome.

1.4.1 Slowly growing bacterial cells.

The use of a recycling fermenter, in which nutrients are added and spent fermentation medium is removed at the same rate, but without the concomitant removal of biomass, has enabled Chesbro *et al.* (1979) to study *E. coli* cells at very long generation times. They have found that *E. coli*, as well as *Paracoccus denitrificans* (Van Verseveld *et al.*, 1984) and *Bacillus polymyxa* (Arbige & Chesbro, 1982 a) showed three well defined growth phases. The first phase is characterised by exponential growth, which ceases when the glucose concentration falls below the cells'

transport threshold. In phase two, the rate of growth is dependent on the rate of glucose provision to the fermenter, and is linear. During this phase the intracellular concentration of ppGpp increases to reach a maximum value. In the third phase there is a slower rate of linear growth, also dependent on the glucose provision rate, and the cells are characterised by being stringently controlled, that is, there is a restriction in the rate of RNA accumulation (Arbige & Chesbro, 1982b). This phase lasts indefinitely.

The change from phase 2 to phase 3 appears to be controlled by the concentration of ppGpp, which reaches levels similar to that in cells in carbon/energy source downshifts, and mutations affecting the concentration of ppGpp (relA, relX, spoT) change the length of phase 2 in a precise manner correlating with the mutational effect on ppGpp concentration (Arbige & Chesbro, 1982 b). Growth during the first two phases is balanced, that is, intracellular components increase in the same proportions. However, during the third growth phase, growth is unbalanced in that RNA does not increase in proportion with other cell constituents (review, van Verseveld et al., 1984).

The level of ppGpp rises continuously throughout phase 2, but the cells do not show stringent control. They may, however, be responding to the increase in ppGpp concentration in ways other than the restriction of RNA synthesis. For example, the cells grow progressively larger as the division rate slows through an increase in cell diameter (Chesbro et al., 1979). This appears to be by changes in the enzymes of phospholipid and peptidoglycan synthesis, which are susceptible to ppGpp regulation (Gallant, 1979).

In both phases 2 and 3, 40 to 60% as much assimilated glucose carbon was secreted, or sloughed (as orcinol, diphenylamine and Lowry reactive material), as appeared as biomass.

1.5. Intracellular protein degradation in bacteria.

In growing bacteria, including thermophiles (Epstein & Grossowicz, 1969) and bacteria capable of sporulation (Urba, 1959; Chaloupka & Strnadova, 1980; 1982), approximately 1 to 2% of the cell proteins are degraded to acid soluble products per h (reviews, Pine, 1972; Goldberg and Dice, 1974; Goldberg & St. John, 1976). Higher rates have been reported (Pine, 1970), and Holzer *et al.* (1975) give a large range (1 to 8% per h) for turnover in growing cells. Some studies suggest that microorganisms living in extreme environments may have higher protein turnover rates than mesophiles. For example, proteolysis in the moderate halophile *Vibrio costicola* is greater than that in *E. coli*, and is affected by changes in salinity (Hipkiss *et al.*, 1980), while protein turnover in a psychrotroph, *Arthrobacter* S_1-55 was low when the cells were grown at low temperature, but high when the cells were grown at the maximal growth temperature for the organism, 32 ^{o}C (Potier *et al.*, 1985). These values (1 to 4 % for mesophiles) resemble those reported for mammalian cells (Schimke, 1973). However, these values were obtained by measuring the degradation into acid soluble material of pre-incorporated radiolabelled amino acids from intact cells, and they cannot distinguish the slow degradation of a large number of proteins from a more rapid loss of label from a small number of proteins.

As the degradation rates of different proteins varies, it is possible to preferentially label groups of proteins with different stabilities by varying the time of pulse labelling (Goldberg & Dice, 1974). By using such a protocol, two kinetically distinct classes of proteins in bacteria can be distinguished, a rapidly turned over fraction, and a more stable one (Pine, 1966; Willets, 1967;Sussman & Gilvarg,1969; Pine, 1970; Chaloupka & Strnadova, 1982).

The labile fraction has been estimated to be about 1 to 7% of the total cell protein (Nath & Koch, 1970; Pine, 1972) and as the bulk of cell proteins are stable, a greater portion of the protein currently being synthesised must be labile. The identity of the proteins in the rapidly turned over fraction is unknown, and attempts to identify them by using two dimensional gel chromatography has lead to unexpected results. Larrabee et al. (1980) were able to separate about 250 soluble proteins from exponentially growing E. coli cells, and using a double label pulse chase technique, found only three proteins that were degraded to any appreciable extent, the vast majority of proteins remaining stable. However, in a study using a similar technique, Mosteller et al. (1980) found that of the 184 proteins they resolved from exponentially growing E. coli cells, 47 were unstable (either modified or degraded) with half-lives varying from 2 to 23 h. The identity of three proteins (involved in tryptophan biosynthesis) were known, of which one (the trpA gene product) is stable, another, the trpB gene product is modified, and the third, the C gene product is unstable. This last, bifunctional enzyme has been shown to be unstable in non-growing cells (Mosteller et al., 1977). Three classes of proteins are identifiable using this

technique, a stable, an unstable, and a modified (modified with respect to molecular mass or iso-electric point) class.

Of the modified proteins, the authors found several pairs of proteins that appeared related to each other as precursor/product, the modification of at least one pair occurring via a proteolytic cleavage.

Regulation of protein degradation would appear to involve changes in the size of the labile pool, rather than a change in the rate of degradation of either the labile or relatively stable pool (Chaloupka & Strnadova, 1982), although the degradation rates of the 'stable' fraction do change marginally. Nath & Koch (1971) have estimated that the size of the labile fraction (with a half-life of 20 to 60 min) increases to 40% of the newly synthesised proteins in slowly growing cells, which means that proteins synthesised during stationary phase or starvation (Pine, 1965; 1972) are more unstable than the original cell proteins, although the enhanced rate of proteolysis during starvation must initially at least, reflect an accelerated degradation of proteins that are stable in growing cells (Pine, 1966; Sussman & Gilvarg, 1969; Pine, 1970). Of the proteins synthesised during growth, 70% are not subject to degradation even after prolonged starvation (Pine, 1973) and protein degradation during starvation continues at an elevated rate for only 6 to 8 h, after which the rate decreases. One factor not controlled in many of these reports is cell viability (Goldberg & St. John, 1976), and this may affect the interpretation of the results.

Wild type E. coli lose 50% viability in approximately 6 d on glucose starvation, whereas mutants lacking combinations of various peptidases

peptidases lost viability more rapidly, indicating that protein degradation has a role in the survival of cells during starvation (Reeve et al., 1984).

Protein degradation in bacteria therefore appears to be fundamentally different from that occurring in mammalian tissues. Almost all intracellular proteins are continuously turned over in mammalian cells (Schimke, 1973), albeit at widely differing rates, whereas proteins in bacteria are generally stable. Inducible proteins are generally not degraded even after removal of the inducer (Mandelstam, 1960), and in general it would seem that the level of any inducible enzyme in bacteria is fixed by the synthesis rate, the enzyme activity on removal of the inducer being diluted only by growth. The particular cause of starvation may influence degradation. For example, beta-galactosidase is lost from E. coli subjected to NH_4^+ ion or leucine starvation (Willetts, 1967), while other enzymes (alkaline phosphatase, D-serine deaminase) are not.

1.5.1. Control of intracellular protein degradation.

The rate of intracellular protein degradation is reversibly regulated. For example, it is enhanced when E. coli is starved of required amino acids, nitrogen or glucose (Mandelstam, 1958; 1960), on entering stationary phase, in the lag period during diauxic growth (Willetts, 1965), or on starvation of various inorganic nutrients (Willetts, 1967; Nath & Kock, 1971; St. John & Goldberg, 1980). Proteolysis increases to

a similar extent (4 to 5%) in E. coli irrespective of the stimulus, and the effect is not additive, suggesting a common control mechanism.

The highly phosphorylated nucleotide, guanosine -5'- diphosphate -3'- diphosphate (ppGpp, also called 'magic spot') has been implicated in the regulation of protein degradation in E. coli (review, Goldberg & St. John, 1976). This regulation appears to be controled by the stringent control system, via the relA gene product (Cashel, 1975), as the lack of a required amino acyl - tRNA specifically causes a concomitant increase in the rate of protein degradation and accumulation of ppGpp (St. John et al., 1978).

Voellmy & Goldberg, (1980) have shown that the rate of intracellular proteolysis is precisely regulated by (or with) the level of ppGpp. They used a technique which enabled them to measure proteolysis over short periods, as the concentration of ppGpp changes rapidly. The level of ppGpp was varied by using different amounts of tetracycline (which directly inhibits the synthesis of ppGpp) (Block & Haseltine, 1974), and the level of proteolysis was found to be proportional to the concentration of ppGpp. However, there must be other factors involved, as the change in the rate of protein degradation in response to a change in the level of ppGpp is different depending whether the stimulus is energy restriction or carbon source starvation (Voellmy & Goldberg, 1980).

1.5.2. Steps in the intracellular degradation of abnormal protein.

Wild type beta-galactosidase (product of the *lacZ* gene) and lactose operon repressor protein (product of the *lacI* gene) are both stable in growing *E. coli* (Rotman & Spiegelman, 1954). However, nonsense polypeptides derived from these genes are present in the cell in smaller numbers than wild type monomers, even though they are synthesised at the same rate (Morrison & Zipser, 1970). Proteins resolved on SDS-polyacrylamide gels after pulse-chase experiments show that the *lacZ* nonsense fragment, X90 (with a mass slightly less than wild type protein) is synthesised with a half-life of approximately 7 min (Goldschmidt, 1970). Furthermore, the initial step in the X90 degradation appears to be an endoproteolytic cleavage, rather than exoproteolytic activity on the ends of the polypeptide chain. The peak width of X90 on the gels remains constant, while the peak height decreases. Furthermore, X90 disappearance is correlated with the appearance of an unstable band of lower M_r and these results suggest a precursor-product relationship. A similar result for the nonsense protein (LI) produced from the *lacI* gene by the L1 mutation (Platt *et al.*, 1970) was found using antibody to the wild type repressor. In this case, no smaller pieces of cross-reacting material were found, although the L1 peak on polyacrylamide gels was always sharp, again suggesting an initial endoproteolytic cleavage, followed by rapid degradation of the products. Kowit & Goldberg (1977) used wild type and *deg*$^-$ mutants of *E. coli* (which have a reduced ability to degrade beta-galactosidase nonsense fragments (Bukhari & Zipser, 1973)) to follow the intermediate steps in the degradation of X90 fragment. They found that the X90 (M_r 120×10^3) fragment is internally cleaved to a more stable polypeptide,

Fragment B (M_r 90 x 10^3), which has the amino terminus of beta galactosidase (as shown by auto alpha complementation). This process was inhibited in *deg*$^-$ cells. They found no other intermediates in the degradation of X90, which may be due to cleavage in the region. Furthermore, the initial cleavage of X90 (half-life 7 min) was much more rapid than the loss of Fragment B (half-life 30 min), so that the disappearance of Fragment B is the rate limiting step in X90 degradation. The authors further showed that the degradation of these auto alpha fragments requires metabolic energy, in that both azide and cyanide (which inhibit respiration), reduce both the conversion of X90 to Fragment B, and the subsequent hydrolysis of Fragment B. This means that energy is required for at least two and possibly more steps in the degradation of abnormal proteins.

The requirement for metabolic energy may be surprising, as the hydrolysis of peptide bonds should, on thermodynamic grounds, yield energy. However, such a requirement is well established (Goldberg, 1972 a; Olden & Goldberg, 1978; St. John & Goldberg, 1978).

Energy-dependent proteolysis specifically required ATP, as the 'energy-rich membrane state' was not sufficient, and, the ATP level had to be reduced to very low levels before protein degradation was inhibited. Furthermore, a moderate decrease in the level of ATP (30 to 50%) actually increased intracellular protein degradation 2-fold, and ATP had to be reduced by over 85% before protein degradation was inhibited (St. John & Goldberg, 1978). Starvation for glucose, amino acids or inorganic ions or changes in cell growth rate caused little or no change in the rate of degradation of abnormal proteins (review,

Goldberg & St. John, 1976), although abnormal proteins were degraded at a higher rate than normal proteins (Goldberg, 1972 a). The difference in the degradative rates of individual proteins, whether normal or abnormal probably reflects differences in their inherent protease susceptibility (Goldberg, 1972 b; Goldberg & Dice, 1974), as is the case in reticulocyte lysates (Ayukawa et al., 1981).

It has been suggested (Goldberg, 1971; Goldberg, 1972 a) that E. coli contains two protein degradative systems. One that is present at all stages, and degrades abnormal proteins, the other being adaptive, that is activated upon starvation or 'hard times'. Certainly in sporulating bacteria, the activation of a separate proteolytic system during sporulation has been shown (1.5.3) however, the position in E. coli remains unclear.

During exposure to the amino acid analogue, canavanine, E. coli produces several large intracellular electron dense bodies. These appear to be attached to the cell membrane, and they have an irregular, amorphous structure (Schachtele et al., 1968). These bodies rapidly sediment, (at 10 000 g) and are not enclosed by a membrane (that is, are not analogous to eukaryotic lysosomes). They are composed of protein of the same amino acid composition as E. coli, but for the presence of canavanine. Prouty & Goldberg (1972) found that the abnormal (canavanyl) proteins accumulated in the 10 000 g pellet prior to degradation, and the subsequent selective loss of canavanyl protein from this fraction was energy-dependent. However, Kemshead & Hipkiss (1974) found that while puromycin and canavanine treatment did increase the radioactivity in the 25 000 g sedimenting fraction, the puromycin peptides were degraded in

the fraction soluble at 150 000 g, and canavanyl proteins were degraded in both fractions. This suggests that the accumulation of abnormal protein in intracellular granules appears to be a spontaneous precipitate of abnormal proteins, rather than being formed by an active process (Prouty et al., 1975), and they appear when the cells' degradative capacity has been exceeded. On removal of the amino acid analogue, the granules disappear, 50% of canavanyl proteins become acid soluble within 1 h (Prouty et al., 1975). There is a possibility that these granules become specifically attached to the membrane, where it has been shown (Voellmy & Goldberg, 1981) that there is a membrane-associated ATP stimulated endoprotease, which rapidly degrades radiolabelled apohaemoglobin.

1.5.3. Protein degradation during sporulation in bacteria.

As in E. coli, the rate of degradation of normal proteins in Bacillus sp. is low during exponential growth (Spudich & Kornberg, 1968), but increases markedly to 7 to 22 % h^{-1} during sporulation (Holzer et al., 1975). The kinetics of protein turnover in growing Bacillus megaterium cells shows the presence of a labile protein fraction decaying with a half-life of less than 1 h, the remaining fraction having a half-life of 40 h or more (Chaloupka & Strnadova, 1982). The rate of degradation of the labile fraction does not change significantly with change in growth rate, and the main target of protein turnover regulation seems to be the relative size of the labile fraction.

During sporulation, the activity of both intracellular and extracellular proteases increases, and a specific intracellular protease inhibitor also appears in these cells (Doi, 1972). The turnover during sporulation is nonspecific (Spudich & Kornberg, 1968), in that newly synthesised protein is degraded at the same rate as pre-existing vegetative cell protein. This general and extremely active protein degradation requires de novo protein synthesis after the end of exponential growth, but before turnover begins. However, once turnover commences, protein degradation continues even if protein synthesis is blocked (Spudich & Kornberg, 1968). As a result of turnover, mother cell protein is extensively degraded and resynthesised (Mandelstam & Waites, 1968), so that total cell protein has been turned over at least twice (Holzer et al., 1975) by the end of sporulation.

Both the intracellular and extracellular proteases appear to be under catabolite repression control (Bernlohr & Clark, 1971; Holzer et al., 1975). The relationship between these enzymes is unclear. Mandelstam and Waites (1968) suggest that the same protease appears to be responsible for both intracellular and extracellular proteolytic activity, and the intracellular serine protease of B. subtilis shows sequence homology with extracellular subtilisins (Strongin et al., 1978). Several enzymes, for example phosphoribosylpyrophosphate aminotransferase (Maurizi et al., 1978; Meyer & Switzer, 1979), are specifically degraded prior to sporulation at rates many fold higher than general protein degradation.

Protein processing also occurs during sporulation. A precursor (M_r 25 x 10^3) of the major sporecoat protein (M_r 13 x 10^3) of B. subtilis has

been shown to be processed both *in vivo* (Munoz *et al.*, 1978) and in a cell-free system (Nakayama *et al.*, 1978). A similar system has been shown in *Bacillus cereus* (Cheng & Aronson, 1977), where the protease clearly has a major physiological role in sporulation. A temperature-sensitive *B. cereus* mutant with a reduced rate of processing and protein turnover produces spores that germinate poorly at high temperature.

Proteolysis also plays a role in germination. Late in sporulation, small proteins are synthesised that eventually comprise about 20% of the protein in the spore. During germination, these proteins are specifically degraded by at least two proteases in the spore coat (Setlow, 1978 a; 1978 b), to provide a pool of amino acids for synthesis of new protein in the germinating spores. These proteases have been purified and have a high M_r. As the cells progress to vegetative growth, both activities disappear.

Encystment in the nitrogen fixing bacterium *Azotobacter vinelandii* shows a pattern similar to sporulation in *Bacillus* species in that extensive protein turnover and amino acid catabolism occurs as new cyst proteins are synthesised. Nitrogen fixation does not occur in *A. vinelandii* during encystment and the initial stages of cyst germination (Sadoff, 1975; Sadoff *et al.*, 1971), however, protein synthesis continues, presumably using amino acids supplied by extensive (50%) turnover during encystment and turnover (7%) during germination (Ruppen *et al.*, 1983). Protein turnover during cyst germination occurs even in the presence of casamino acids, which are readily taken up, and may be due to a need to remove certain cyst-specific proteins from the differentiating cells.

1.5.4. Intracellular proteases and esterases in E. coli and related Gram-negative bacteria.

There are at least eight proteases in E. coli (Swamy & Goldberg, 1981) and possibly many more (Pacaud, 1982). The uncertainty is caused by the choice of substrates used in assaying these activities, and the low intracellular concentration of these activities.

Proteases such as I, II and ISP-L-Ecs (Pacaud et al., 1976; Strongin et al., 1979) were identified and purified by their ability to hydrolyse chromogenic ester substrates (N-substituted peptides). However, some of these proteases show little or no activity against classical protease substrates, such as casein (Kowit et al., 1976). Furthermore, S. typhimurium mutants deficient in both proteases I and II have been isolated (Heiman & Miller, 1978 b) and neither growth nor the ability to degrade proteins (abnormal or normal during carbon-source starvation, was affected. The role of these activities remains unknown.

Swamy & Goldberg (1981; 1982) used radiolabelled proteins, casein, apohaemoglobin (globin) and insulin (Goldberg et al., 1981) to isolate and establish the subcellular location of eight proteases named Pi (periplasmic, insulin-degrading) Ci (cytoplasmic, insulin-degrading) and serine proteases Do, Re, Mi, Fa, So, La (these last named in the order of elution from DEAE-cellulose during purification).

One of these, La, is ATP-dependent (Swamy & Goldberg, 1981) and is the product of the lon gene (synonyms; deg, capR; Chung & Goldberg, 1982). La is an unusual enzyme in that it is a serine protease, an ATPase and binds DNA. The inhibition of ATP hydrolysis prevents proteolysis, which possibly accounts for the energy requirement for protein degradation in whole cells (Olden & Goldberg, 1978).

The lon mutation has a pleiotropic phenotypic effect, causing increased sensitivity to DNA damaging agents such as ionising radiation, U.V. radiation and methyl methane sulphonate, probably by preventing the inhibition of filamentation controlled by the sulA locus (Mizusawa & Gottesman, 1983), decreased ability to degrade abnormal proteins (Shineberg & Zipser, 1973; Murakami et al., 1979) and over production of capsular polysaccharide (Markovitz, 1977). Furthermore, La binds DNA with high affinity, possibly regulating gene expression (Markovitz, 1977) through an alteration of proteolytic specificity (Chung & Goldberg, 1982).

Protease Pi has been shown to be identical to protease III (Cheng & Zipser, 1979) which is a product of the ptr gene. It is a periplasmic protein, with two gene products, a 110 x 10^3 dalton protein, and a second 50 x 10^3 dalton polypeptide apparently derived from the amino terminal end of the coding sequence, which does not appear to originate from autolysis of the larger protein, and does not show proteolytic activity (Dykstra & Kushner, 1985).

Protease III is probably not essential for normal cell growth since

neither deletion of the structural gene nor a 30-fold over-production of the protease affected the phenotype (Dykstra & Kushner, 1985).

Protease III is restricted to the periplasm (Cheng & Zipser, 1979; Swamy & Goldberg, 1981), which suggests a role in membrane protein processing, or the hydrolysis of exogenous substrates (Heppel, 1971). This last role may explain why loss of this activity had no detectable phenotypic alteration (Cheng et al., 1979).

Of the other seven activities identified using these substrates, Mi is restricted to the cytoplasm, Re, a serine protease, is found equally distributed between the cytoplasm and periplasm, and the other five serine proteases are found in the cytoplasm (Swamy & Goldberg, 1982).

1.5.5. ATP-dependent proteolysis in reticulocytes.

Reticulocytes have a non-lysosomal heat stable ATP-dependent proteolytic activity which is responsible for the selective degradation of abnormal proteins, as well as the loss of organelles occurring during reticulocyte maturation (Etlinger & Goldberg, 1977; review, Hershko & Ciechanover, 1982). The ATP-dependent proteolytic activity declines with reticulocyte maturation and decreases further to negligible levels with erythrocyte ageing. This age-dependent loss may result in the toxic accumulation of oxidised and abnormal proteins, influencing the life time of erythrocytes (Speiser & Etlinger, 1982).

The role of ATP in reticulocyte proteolysis is not well understood. Boches et al. (1980) purified an ATP-stimulated endoprotease (M_r 470 x 10^3) from reticulocytes, which is stimulated two to three times by 1 mM ATP, and is also stimulated by other nucleotide phosphates and inorganic pyrophosphate. This serine protease cleaves globin to fragments larger than 1,5 x 10^3 daltons, and the authors propose that this endoprotease catalyses the rate limiting step in the breakdown of abnormal proteins.

Hershko et al. (1980) (reviews, Hershko & Ciechanover, 1982; Finley & Varshavsky, 1985) propose that an ATP-dependent, heat stable protease factor (APF-1, since shown to be ubiquitin) of reticulocytes forms covalent compounds with protease substrates in an ATP-requiring reaction, and that this step is essential for the selective degradation of intracellular proteins. Such complexes appear to form from covalent bond formation between the E-amino groups on proteins and the carboxy terminus of ubiquitin. Presumably the protease(s) recognise this complex for subsequent degradation.

The role of ubiquitin still remains unclear, and recently Waxman et al. (1985) have presented evidence for a protease which requires ATP hydrolysis but not ubiquitin from murein erythroleukaemia cells.

Speiser & Etlinger (1983) have purified an endogenous inhibitor of the ATP-dependent system, and propose that the inhibitor-free protease can degrade substrates such as [^{14}C]-methyl alpha casein. The inhibitor can be repressed by ATP if ubiquitin is present, thus stimulating endoproteolysis. With reticulocyte maturation, the inhibitor and

protease remain, but the ubiquitin fraction is less effective in repressing the inhibitor.

1.5.6. The RecA proteolytic system.

RecA protein, the product of the recA gene, is a multi-functional protein present in E. coli. It catalyses several novel DNA reactions, such as annealing of single stranded DNA (review, Radding, 1981), it has a DNA-dependent ATPase activity, and a proteolytic activity. RecA protein is essential for genetic recombination in E. coli, it regulates the inducible SOS pathway of DNA repair and in vitro it promotes homologous pairing of DNA molecules (review, Little & Mount, 1982).

The primary event that triggers the SOS response is the reversible activation of the RecA proteolytic activity. This in turn cleaves the lexA gene product, LexA, which represses at least 9 genes, including itself and recA. It also cleaves the lysogenic phage A repressor, inducing the lysogen (Roberts et al., 1978; Craig & Roberts, 1980; Phizicky & Roberts, 1980; 1981). Both Lex A and its repressor monomers have two functional and physical domains, the amino terminal domain, that determines operator recognition, and a carboxy terminal domain that provides most of the contact for dimer formation. Both proteins exist as dimers, and bind to operator sequences which have dyad symmetry, thus excluding RNA polymerase (Little & Mount, 1982). In all but lexA operator, LexA protein binds a 25 bp region (45 bp long in LexA operator) these all have a 20 bp consensus sequence, called the 'SOS box'.

RecA cleaves at an -ala-gly- sequence near the middle of both LexA and repressor (Horii et al., 1981), in a 'hinge' region between the functional domains. The amino acid sequences near the sites have some homology, suggesting that the protease may recognise a distinct tertiary structure, explaining its very high specificity.

The proteolytic activity requires Mg^{2+}, as well as the following; a polynucleotide co-factor, the specific substrate, and any one of these triphosphates, dATP (the deoxy ribonucleotide), ATP, or an analogue, ATP-γ-S. Both dATP and ATP-γ-S stimulate a higher rate of reaction than does ATP, suggesting that the nucleotide acts as an allosteric effector (Craig & Roberts, 1980). The polynucleotide requirement can be fulfilled by a variety of single stranded polynucleotides in vitro. There is an optimum ratio of 4 polynucleotide strands per RecA monomer. LexA cleavage is markedly and specifically inhibited in the presence of DNA containing recA operator sequences, which suggests that the repressor must be dissociated from DNA for cleavage.

1.5.7. The role of peptidases in intracellular proteolysis.

E. coli and S. typhimurium mutants deficient in peptidases have been obtained by selecting for the inability to hydrolyse chromogenic peptide substrates, resistance to peptides containing a toxic amino acid, or utilisation of small peptides for growth (Miller & Schwartz, 1978). The loci pepB, pepD and pepN control the synthesis of four peptidases with broad overlapping substrate specificities (Miller & Mackinnon, 1974;

Kirsh *et al.*, 1978). The loci *pepP* and *pepQ* specify two proline specific peptidases (McHugh & Miller, 1974). The locus *apeA* specifies protease 1, active towards ester substrates of chymotrypsin (Pacaud *et al.*, 1976; Heiman & Miller, 1978 a), and *tlp* specifies a trypsin-like activity, protease II, which hydrolyses peptide substrates of trypsin (Pacaud & Richaud, 1975), but is not very active towards protein substrates (Pacaud, 1976). Protease I is located primarily in the periplasm, has been purified, and found to have very weak activity against proteins, necessitating long incubation times. Indeed the proteolytic function of this enzyme has been questioned (Kowit *et al.*, 1976) and the proteolytic activity may be due to a second contaminating protease.

None of these activities seem to be involved in the degradation of large protein fragments initially generated by endoproteolysis, as mutants lacking peptidases A, B, N, P, Q or proteases I & II show the normal rapid degradation of beta-galactosidase nonsense fragments (Miller & Zipser, 1977).

However, these mutants do accumulate a heterogenous mixture of small TCA soluble peptides during growth in minimal medium (Yen *et al.*, 1980a). Approximately 20% of radiolabelled leucine supplied to the cell accumulates in the cell as peptides before being released into the medium. Some of these peptidases also function in the utilisation of exogenously supplied peptides (Kirsh *et al.*, 1979). The origin of these peptides is presumed to include the turnover of signal sequences, attenuator peptides and peptides produced by endo-proteolysis of abnormal proteins.

The degradation of abnormal proteins produced as a result of the incorporation of L-canavanine (an arginine analogue) or puromycin (causing the premature release of incomplete nascent polypeptides) was followed in both wild type and peptidase-deficient S. typhimurium (Miller & Green, 1981). The wild type strain produced free amino acids from the breakdown of abnormal proteins, whereas the mutant yielded a complex mixture of small peptides and free amino acids, indicating that peptidases are involved in the degradation of abnormal proteins. Furthermore, during starvation of a carbon source, when protein turnover increases markedly (Goldberg & St. John, 1976) multiply peptidase-deficient mutants of S. typhimurium fail to carry out normal protein degradation. The extent of protein breakdown is 4-fold less than in the wild type (Yen et al., 1980) and the degradation products are mainly small acid-soluble peptides, not free amino acids. This means that intracellular pools of amino acids are smaller in the mutant, and protein synthesis during starvation is reduced, which causes the mutants to show prolonged lag phases after nutritional shift down.

The impaired protein turnover affects the survival of bacteria on starvation. E. coli K-12, starved for glucose, loses 50% viability in approximately 6 d (Reeve et al., 1984). However, peptidase deficient mutants of E. coli K-12, lose viability more rapidly, those lacking five peptidases lose 50% viability in approximately 2 d. S. typhimurium mutants lacking various combinations of the 3 or 4 peptidases show protein degradation and synthesis rates intermediate between wild type and the mutant deficient in 5 peptidases (Yen et al., 1980).

1.5.8. Membrane bound and membrane associated proteolytic activities.

Proteins that are secreted across or become integrated into cell membranes in both prokaryotic and eukaryotic cells are often synthesised as longer precursor molecules, the amino terminal end of such proteins possessing a signal sequence. In the signal hypothesis (Milstein et al., 1972; Blobel & Dobberstein, 1975), it is proposed that in eukaryotic cells the precursors are synthesised by membrane attached ribosomes, and that transport of the precursors across the membrane occurs concurrently with translation. The signal sequence conducts the protein through the membrane and is then removed proteolytically during or after secretion (Lingappa et al., 1977). Such a mechanism does not account for all of the features of protein secretion in bacteria (Wickner, 1979), and in the membrane trigger hypothesis it is suggested that membrane associated or secreted proteins are initially synthesised in the cytoplasm. The signal sequence influences initial polypeptide conformation, allowing the hydrophobic regions of the polypeptide to interact with the cytoplasmic membrane. Signal sequence removal releases the secreted protein or makes it integrate into the membrane.

A third model has been proposed (Di Rirenzo et al., 1978) which is similar to the signal hypothesis, but in this model the preprotein forms a loop at the signal peptide, which passes through the membrane, and is then cleaved on the outside surface. Chang et al. (1980) have shown that both inner and outer membrane fractions of E. coli can process the precursor form of alkaline phosphatase in vitro. Zwisinski et al.

(1981) found a preprotein processing activity, leader peptidase, in equal abundance in both the inner and outer membranes, which accurately cleaved phage M13 procoat protein to its mature form. Tokunaga et al. (1982) have reported a further signal peptidase (prolipoprotein signal peptidase) that is distinct from the M13 procoat protein signal peptidase, and is located exclusively in the inner membrane. Using chromogenic substrates N-acyl-amino acid-p-nitrophenyl ester because of their sensitivity and specificity, Pacaud (1982) isolated two membrane bound esterases, called protease IV and V. Protease IV is localised in the cytoplasmic membrane, whilst protease V is found in both the inner and outer membranes.

Signal sequences undergo two successive proteolytic attacks, cleavage of the signal peptide from the pre-protein by signal peptidase, followed by digestion of the signal peptide, by a signal peptide peptidase. Protease IV has been shown to have signal peptide peptidase activity (Ichihara et al., 1984). This activity, which is integrated in the membrane (Pacaud, 1982) attacks the signal peptide only after its release from the precursor protein (Ichihara et al., 1984).

Signal peptides have a variety of amino acid sequences including different sequences at the cleavage site (Davis & Tai, 1980). It would be of great interest to know if signal sequences in E. coli are cleaved by the two known signal peptidases, or whether there are other activities present. M13 procoat protein peptidase is able to cleave the precursor forms of two periplasmic amino acid-binding proteins of the outer membrane (quoted by Zwisinski et al., 1981).

Voellmy & Goldberg (1981) have shown that an ATP-stimulated endoprotease (active against globin or denatured E. coli proteins) is associated with the cell membrane. This activity degrades proteins to peptides of M_r 1 500 daltons and larger. The relationship of this activity to the soluble ATP-stimulated cytoplasmic protease La remains unclear.

In addition to the activities mentioned, several proteolytic activities have been found associated with the E. coli outer membrane. MacGregor et al. (1979) demonstrated a [^{3}H]-DFP (diisopropyl fluorophosphate) binding activity that cleaved one of the nitrate reductase subunits on heating membrane preparations to 60 °C for 10 m in alkaline conditions. They found one [^{3}H]-DFP-binding activity in the outer cell membrane, and suggested that this was responsible for the processing of outer membrane proteins during assembly. Growth of the cells in p-amino benzamidine (pAB) inhibited this activity, and affected the synthesis of outer membrane proteins, but not inner membrane proteins. Morona & Reeves (1984) have shown that there are several [^{3}H]-DFP-binding proteins (presumably serine proteases) in the E. coli K12 outer membrane, the number of activities depending on the particular K12 strain.

Binding of specific receptors in the E. coli outer membrane is the initial step in the killing of sensitive cells (Maeda & Nomura, 1966; Nomura, 1967). Presumably those bacteriocins with intracellular targets, such as E2 and E3 (Nomura et al., 1974; Schaller & Nomura, 1976) cross both cell membranes. However, colicin E1, which destroys membrane potential (Weiss & Luria, 1978) need not penetrate both

membranes, as colicin molecules covalently bound to Sephadex beads can still kill cells (Lau & Richards, 1976). Interaction of colicin E1 with the surface of E. coli cells results in the release of colicin fragments in the M_r range 10 to 30 x 10^3 daltons (Brey, 1982). Proteolytic cleavage has been proposed as being involved in cell killing (Watson & Sherratt, 1979; Cavard & Lazdunski, 1979). However, cleavage of colicin Ia appeared to be independent of receptor function (Bowles & Konisky, 1981) and inhibition of colicin cleavage by PAB (Brey, 1982) enhanced colicin killing, suggesting that proteolysis is not essential to colicin A action.

The proteolytic activity that cleaved colicin A may have a second function, that of an osmotic sensor in the outer membrane (Cavard et al., 1982). They proposed that the cpr protease is a product of the envZ gene which is an envelope protein proposed to work as an osmosensor (Hall & Silhavy, 1981 a; 1981 b). Internal osmotic regulation in E. coli is controlled by the products of the locus kdp, all of which are inner membrane proteins. The osmotic pressure in the periplasmic space is regulated by membrane-derived oligosaccharides (Kennedy, 1982). The role as osmoregulator for the outer membrane protease would be a unique role for a protease, but has not yet been established.

1.6. Studies with Vibrio sp.2.

Phage Alpha 3a growth in stationary phase cells was found to be completely inhibited in O_2 concentrations greater than 5,2 p.p.m. (Robb et al., 1977). Removal of the stationary phase cells from aeration

(shaking) for 24 h was found to provide optimal conditions for phage growth. Some morphological and physiological changes that take place in these cells were investigated, and are presented here.

The striking observable difference between shaken and standing stationary phase cells was found to be the enhanced level of protein synthesis (measured as the incorporation of radiolabelled amino acid into acid precipitable material) found in shaken stationary phase cells (Robb *et al.*, 1980). Robb (1979) proposed that this is due to 'sick' uncontrolled protein synthesis, possibly analogous to that seen in 'relaxed' (*relA*$^-$) cells. A study of protein turnover and intracellular proteolysis in *Vibrio* sp. 2 was undertaken.

CHAPTER TWO.

CHARACTERISTICS OF STATIONARY PHASE VIBRIO CELLS.

2.1. Introduction.

Alpha 3a growth in stationary phase Vibrio cells (1.1.1.) was studied in detail by Robb (1979). Optimal conditions for phage development in stationary phase were found to include a 'microaerophilic' environment, that is, the yield of phage in stationary phase cells was affected by the ratio of culture surface area to the culture volume. Phage development was inhibited by either aeration or stringent anaerobic conditions.

Robb (1979) made a comparative study of several characteristics between shaking (aerated) and standing (non-aerated) stationary phase Vibrio cultures. She found that standing stationary phase cells showed increased viability and resistance to heat and uv inactivation, as well as increased levels of intracellular ATP and RNA synthesis, when compared with aerated (shaken) stationary phase cells. Light microscopic investigation suggested that the shaken stationary phase cells appeared small and non-motile, while cells from standing cultures appeared as large, motile cells.

An outstanding characteristic was the difference in macromolecular synthesis measured in the cultures. While neither shaking nor standing stationary phase culture showed consistently measurable DNA synthesis, both protein and RNA synthesis could be measured by following the incorporation of radiolabelled uracil or leucine into trichloroacetic acid (TCA) precipitable material. Shaken cultures showed a high level of protein synthesis compared with standing cultures, while the level of RNA synthesis in shaken cultures was much lower than that measured in standing cultures. Robb (1979) suggested that shaken cultures were 'sick' and exhibited uncontrolled protein synthesis.

Shaken cells were able to adsorb alpha 3a, but the phage DNA was not replicated. However, infected shaken cells could support phage growth when aeration ceased (Woods, 1976).

Bacterial cells do not always exhibit co-ordinately controlled macromolecular synthesis (i.e. they are not always in balanced growth), for example cells that are stringently controlled (1.3.) or cells in phase 2 and phase 3 of very slow growth (Arbige & Chesbro, 1982). During phase 2 of very slow growth (1.4.1.) the levels of both ppGpp and pppGpp rise continuously, until they reach the levels measured in stringently controlled cells.

Morphological changes were also observed in slowly growing E. coli cells (Chesbro et al., 1979) and although the cellular components appeared to change little, there was a general trend towards plasmolysis with culture age, and a distinct progressive change in dimensions as the cells became more coccoid in phases 2 and 3. Cell volumes, computed

from measurements of cell length and width, increased with culture age, while the proportion of dividing cells decreased. Protein turnover, the role of the magic spot nucleotides, pppGpp and ppGpp, and changes in Vibrio morphology that occur as the cells become able to support phage Alpha 3a growth in stationary phase were investigated.

2.2. Materials and methods.

2.2.1. Cultures and media.

Vibrio sp. 2 was grown in tryptone broth according to the conditions given by Robb (1979). Exponential phase cultures were grown at 30 °C with shaking (150 o.p.m. in an orbital shaker). Shaking stationary phase cultures were grown likewise, but for 3 d with shaking. Shaken (2 d) cultures were removed from shaking and then either dispensed in 10 ml aliquots into sterile standard containers with loose caps or dispensed in 3 ml aliquots into 13 x 100 mm sterile glass tubes with plastic caps, and incubated without shaking at 30 °C for 1 d, to give standing stationary phase cultures. These conditions were found to be optimal for the growth (in non-aerated cultures) or inhibition of growth (in aerated cultures) of phage Alpha 3a on stationary phase Vibrio cells (Robb, 1980).

2.2.2. Measurement of RNA and protein synthesis.

Synthesis of these macromolecules in stationary phase Vibrio cells was measured by following the incorporation of radiolabelled leucine and methionine (protein synthesis) and uracil (RNA synthesis) into acid precipitable material. [^{3}H]-Uracil was added to a final concentration of 2 μg ml^{-1}; 0,4 μCi ml^{-1}. [^{14}C]-Leucine was added to a final concentration of 10 μg ml^{-1}, 1 μCi ml^{-1}. [^{35}S]-Methionine was added to a final concentration of 10 μg ml^{-1}, 0,2 μCi ml^{-1}. Samples (0,5 ml) were withdrawn at appropriate intervals and added to an equal volume of ice cold TCA (10 % w/v) containing uracil (1 mg ml^{-1}) or leucine (1 mg ml^{-1}) or methionine (1 mg ml^{-1}) as appropriate. After 30 min on ice, the samples were collected on Whatman GF-C filters, washed twice with 10 ml ice cold TCA (5% w/v) and counted. Cultures were adjusted to the same absorbance (1,6 at 600 nm) when required, with sterile 'spent' medium (Robb, 1979) prior to addition of radiolabel. Control experiments show that Vibrio sp. 2 does not produce extracellular, acid precipitable proteins de novo in 3d shaken or standing cultures.

2.2.3. Measurement of protein turnover rates.

The release of radiolabel into acid soluble material from pre-incorporated radiolabelled amino acid was used as a measure of protein turnover in cells. Protein degradation was expressed as the percentage of radiolabel incorporated into protein that became acid soluble. Cells from an overnight culture were inoculated (to A_{600} = 0,05) into 8 ml of Tryptone Broth containing [^{14}C]-leucine (0,2 μCi ml^{-1}) in 50 ml

erlenmeyer flasks and shaken at 30 °C. Exponential cultures were incubated for at least two generations (growth was followed by monitoring the absorbance of parallel, nonradiolabelled cultures) before harvesting. Aerated stationary phase cultures were incubated for 3 d before the measurement of turnover, except where noted (Fig. 2.6) and non-aerated stationary phase cultures were shaken for 2 d before being dispensed into sterile standard containers and incubated at 30 °C without shaking for 1 d before turnover was measured.

The labelled cells were harvested, washed twice, then resuspended in prewarmed broth with 100 μg ml^{-1} unlabelled leucine added. Stationary phase cells were washed and resuspended in prewarmed 'spent' medium (Robb, 1979) with 100 μg ml^{-1} unlabelled leucine. Duplicate 0,45 ml aliquots were taken at 10 min intervals, added to 50 μl TCA (100% w/v) in microfuge tubes, and mixed immediately.

Samples were left for at least 1 h at room temperature before centrifugation (5 min in an Eppendorf microfuge). Acid soluble radioactivity was determined in 0,25 ml aliquots of the supernatant by liquid scintillation. Initial acid insoluble radioactivity was determined by washing the precipitates of the appropriate samples with 1 ml of cold 5 % (w/v) TCA containing 0,1 % (w/v) BSA as carrier, then removing the TCA by resuspending the pellet in 1 ml of an ice cold dry mixture (1:1 v/v) of ethanol/ether. The precipitates were dried, dissolved in 50 ul of 1 N NaOH then neutralised with 0,1 N HCl and the volume made up to 0,45 ml with water. Radioactivity was determined in 0,25 ml of the dissolved precipitate.

Protein turnover in starving cells was measured as above, however, the prelabelled incubated cultures were washed and resuspended in prewarmed TMS [Tris/HCl,100mM,ph 7,6;NaCl 0,4M; $MgCl_2$ 0,4mM] buffer containing 100 $\mu g\ ml^{-1}$ unlabelled leucine. Protein turnover in cells with blocked protein synthesis was measured as above, however washing and resuspension was performed with medium (either broth, or buffer for starving cells) containing 100 $\mu g\ ml^{-1}$ chloramphenicol (cml).

2.2.4. Detection of ppGpp in Vibrio cells.

The method of extracting nucleotides at pH 7.8 (Lagosky & Chang, 1978) as modified (Lagosky & Chang, 1980) was used. Nucleotides were separated on polyethyleneimine-cellulose thin layer chromatography plates (Cashel & Gallant, 1968).

Vibrio was grown as described (2.2.1). Nucleotide pools were labelled by the addition of carrier-free [^{32}P]-orthophosphate at 30 $\mu Ci\ ml^{-1}$ to 3 ml volumes of culture at the appropriate stage. Exponentially growing cells were labelled for at least two generations. Stationary phase cells were incubated for 2 d with shaking, then radiolabel was added. Aerated stationary phase cells were incubated for a further 24 h with shaking. Non-aerated stationary phase cells were dispensed (3 ml volumes) into sterile 13 x 100 mm glass tubes as described (2.2.1) and incubated without shaking for 24 h at 30 ^{o}C.

Volumes (10 ml) of exponential cells (at approximately 5 x 10^8 cells ml^{-1}), and 1 ml volumes of stationary phase cells (at approximately 5 x 10^9 cells ml^{-1}) were pelleted by centrifugation in pre-chilled tubes and

rotor at 20 000 g, placed on ice and the supernatant removed. The cells were resuspended in 200 µl ice cold LTM buffer (lysozyme 1 mg ml^{-1}, Tris/HCl (pH 7.8) 10 mM, magnesium acetate 15 mM) vortexed, then freeze thawed twice in dry ice/acetone and ice water. A 10 % (w/v) deoxycholate solution (15 µl) was added, the cultures vortexed then centrifuged in an Eppendorf microfuge (5 min). Cell lysis by this procedure was monitored in parallel unlabelled cultures using phase contrast microscopy. Aliquots (1 to 3 µl; where 1 µl represents the extract of 2,3 $x10^7$ cells from exponential and both types of stationary phase cultures) of the supernatant were immediately chromatographed and the rest kept at -70 °C for duplicate runs.

The samples were run on PEI-cellulose (20 x 20 cm) plates (E Merck, Darmstadt). The plates were pre-run in distilled water and dried before use. For one-dimensional chromatography the solvent was 1,5 M KH_2PO_4, pH 3,4 (Cashel et al., 1969). For two dimensional chromatography, the first solvent used was 1,5 M LiCl, the plates were then dried, washed twice by immersion in dry methanol to remove the salt, then dried and developed in 1,5 M KH_2PO_4 (pH 3.4). Radioactivity was detected by autoradiography (Kodak XAR-5 X-Ray film) for one week at -70°C. Spots were identified by using authentic standards (ppGpp and pppGpp were obtained from Sigma), and visualised by u.v. fluorescence.

Control experiments to demonstrate that the nucleotide pools were radiolabelled by [^{32}P] in broth were carried out by measuring the incorporation of [^{32}P] into acid precipitable material as described (2.2.2) but using 10 µCi ml^{-1} [^{32}P]-orthophosphate as the radiotracer.

Accumulation of ppGpp was induced by using 10 mM hydroxylamine (Lund & Kjeldgaard, 1972). Control experiments determining the effect of the addition of 10 mM hydroxylamine on RNA and protein synthesis were performed as described (2.2.2). Samples to be used for the detection of ppGpp were taken 5 min after the addition of hydroxylamine, shown to be optimal for ppGpp induction (Lund & Kjeldgaard, 1972).

2.2.5. Vibrio cell morphology and size.

Thin sections of fixed and imbedded Vibrio cells from the various stages of growth (2.2.1) were examined by transmission electron microscopy. Cell parameters were measured from electron micrographs of negatively stained Vibrio cells.

2.2.5.1. Electron microscopy of thin sections.

Samples of cultures (ca. 10^8 cells) were taken at the appropriate times, and fixed by the method of Kellenberger et al., (1958). The fixed cells were dehydrated using a series of acetone solutions of increasing concentration. The samples were imbedded in NC1010 Spurr low-viscosity resin (Polaron Equipment Ltd., Hertfordshire, England). Sections were cut using a glass knife on an ultramicrotome (LKB Instruments, Inc., Rockville, Md.). The sections were stained in saturated aqueous uranyl acetate for 3 min, washed with water, then stained in lead citrate (0,2 % in 0,1 N NaOH) for 3 min, washed with water, placed on a new untreated grid and viewed in a Zeiss 109 electron microscope at 80 kV..

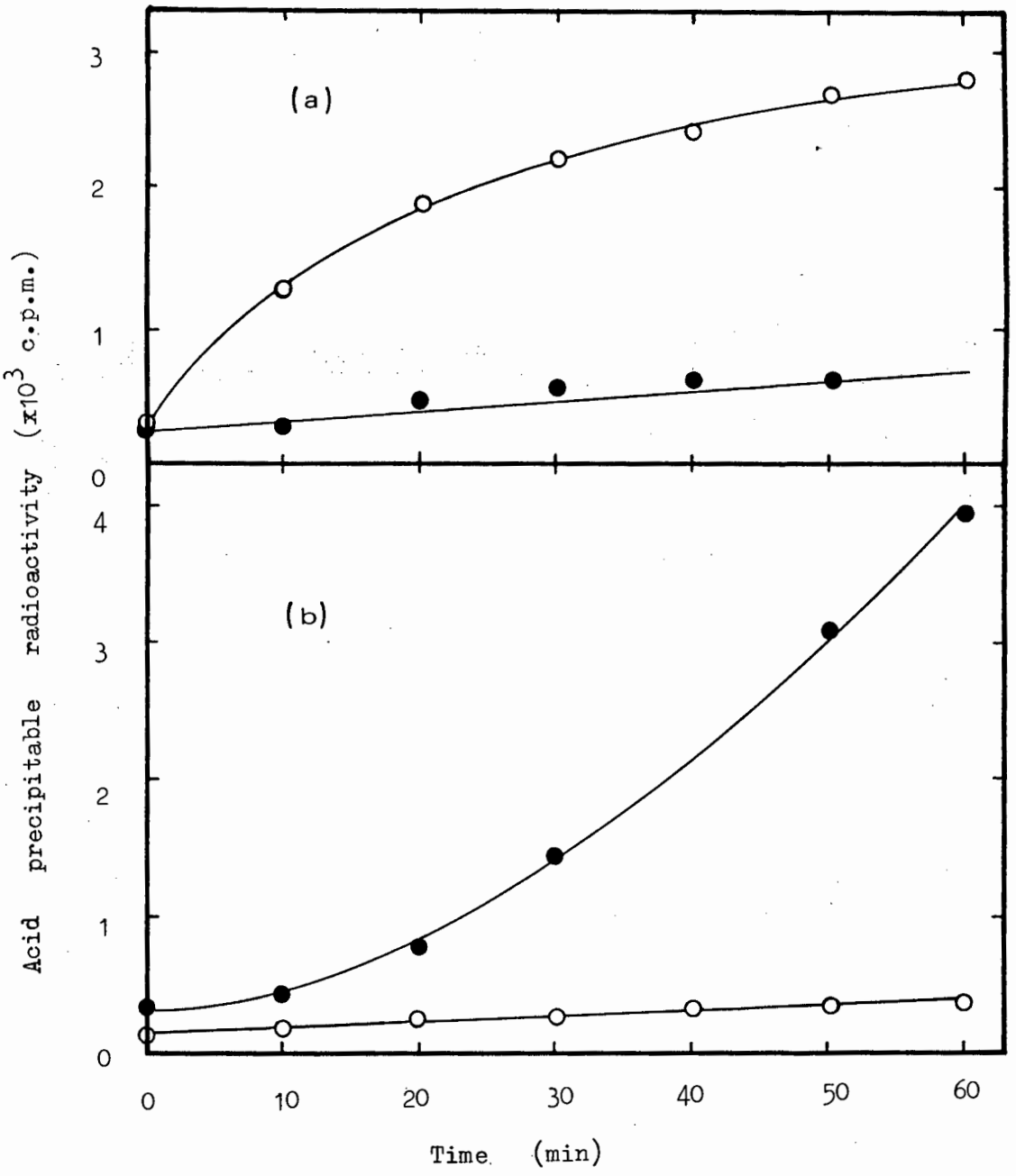


Fig. 2.1. **Incorporation of radiolabel into TCA precipitable material.**

The incorporation of (a) [^{14}C]-leucine and (b) [^{3}H]-uracil into shaking (o), and standing (●) stationary phase *Vibrio* cells was followed as described (2.2.2).

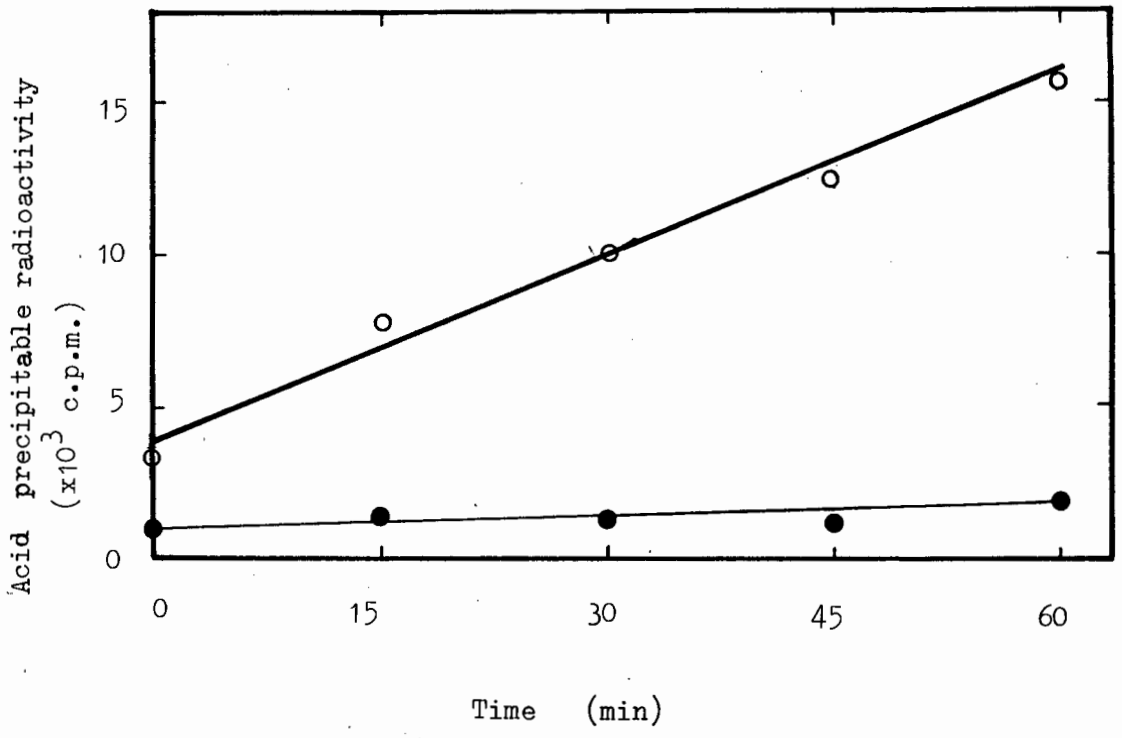


Fig. 2.2. Incorporation of [^{35}S]-methionine into TCA precipitable material.

The incorporation of [^{35}S]-methionine into TCA precipitable material by shaking (o) and standing (•) stationary phase cells was followed as given (2.2.2).

2.2.5.2. Electron microscopy of negatively stained whole cells.

Samples (c. 50 µl) of culture were taken at the various stages of growth and placed directly onto carbon coated formvar grids, left for 4 min and the liquid was then removed by capillary action using filter paper. The sample was stained by adding one drop of 0,5 % (w/v) phosphotungstic acid, pH 7,0 for 1 min and the stain was removed using filter paper. The samples were viewed immediately as no drying was necessary. Representative areas were photographed.

2.3 Results.

2.3.1. RNA and Protein synthesis in stationary phase Vibrio cells.

The results obtained (Fig. 2.1) confirm those established previously (Robb, 1979; Robb et al., 1980). The incorporation of [^{14}C]-leucine into acid precipitable material occurred at a higher rate in aerated stationary phase Vibrio cells, than it did in non-aerated stationary phase cells, whereas the incorporation of [^{3}H]-uracil into TCA precipitable material occurred at a higher rate in non-aerated cultures than in aerated cultures. The difference in amino acid incorporation between the two types of stationary phase cells also applied to [^{35}S]-methionine (Fig. 2.2). The quantity of protein synthesis differed between Vibrio strain 14 used by Robb et al. (1980) and Vibrio sp.2, and there is a difference in the kinetics of [^{14}C]-leucine and [^{3}H]-uracil accumulation between the strains. In both cases the level of protein synthesis is small relative to that in exponential phase cells.

Table 2.1. Protein Turnover Rates in Vibrio.

Protein turnover was measured by measuring the release of radiolabel from prelabelled cells in various growth conditions. Cells were starved by resuspending prelabelled cells in growth limiting medium (2.2.3) prior to turnover measurements. Data from at least 3 independent experiments were used to calculate turnover rates. Standard deviations of the data are given as a percentage of the mean. No single deviation was greater than 8 %, except as mentioned below. Rates were calculated using linear least square regression analysis.

Cells	Turnover ($\%h^{-1}$)	Turnover during starvation ($\%h^{-1}$)	Turnover +CML ($\%h^{-1}$)	Turnover starving + CML ($\%h^{-1}$)
Exponential	1,8	14,2[a] 4,2[b]	6,4	7,7
(% deviation)	2,8	4,4	4.8	3,2
Shaking	1,5	3,0	1,2	0,96
Stationary (% deviation)	3,4	1,1	2,0	2,9
Standing	2,9	0,7[c]	2,1[c]	nmd
Stationary (% deviation)	3,7	-	-	-

(a) initial rate
(b) final rate
(c) deviation in the data was large, see Figs. 2.4; 2.5.
(nmd) no meaningful data could be obtained.
(-) % deviations for these data were not calculated.

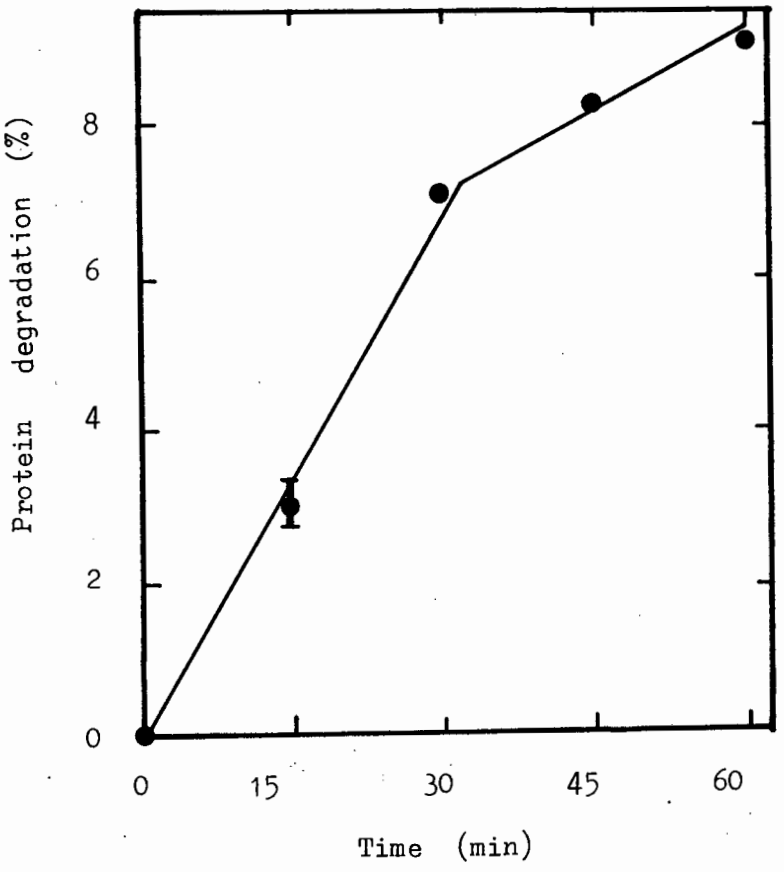


Fig. 2.3. Protein degradation in starving exponential phase Vibrio cells.

Prelabelled exponential phase cells were resuspended in growth limiting medium (2.2.3) and the protein degradation determined. The data were the means of 3 independent experiments. Standard deviation from the mean is given as a bar for the 15 min datum, deviation for the other points was less.

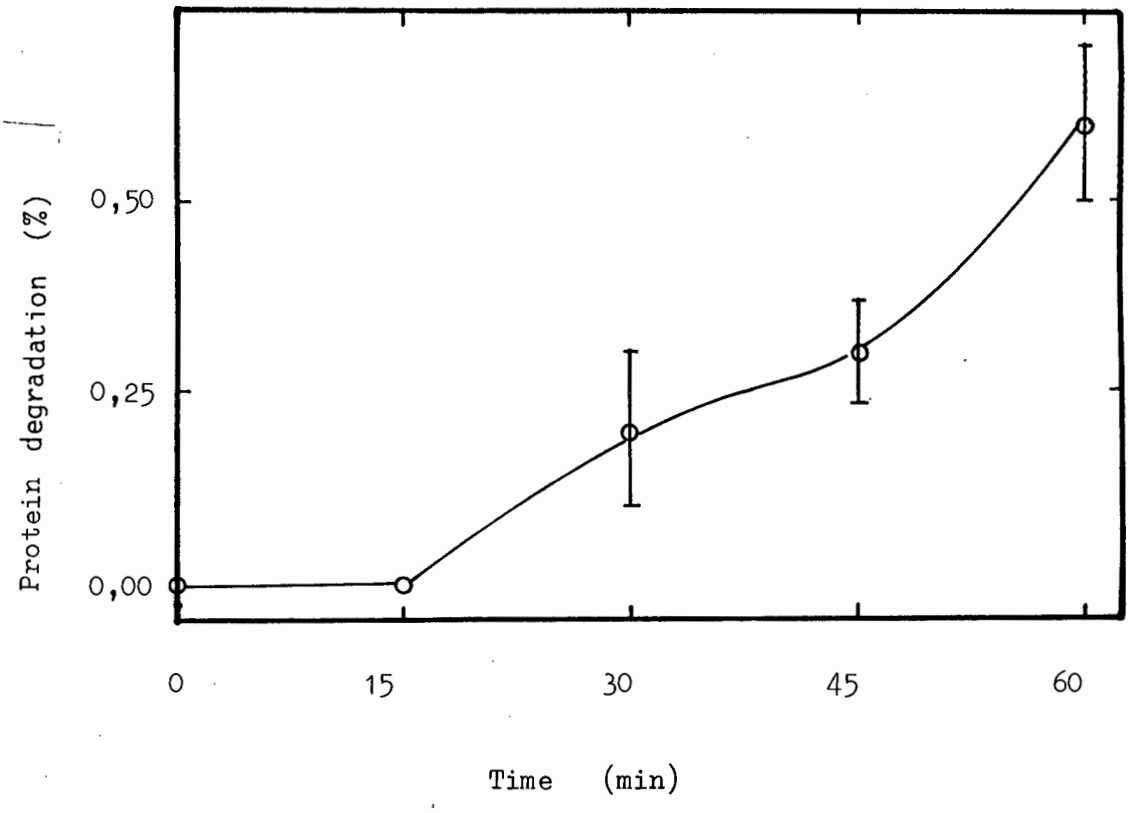


Fig. 2.4. Protein degradation in starving Vibrio standing stationary phase cells.

Prelabelled standing stationary phase cells were resuspended in growth limiting medium (2.2.3) and the protein degradation determined. The data were the means of 4 independent experiments. Standard deviations from the mean are given as bars. The rate of degradation, calculated by regression analysis from data after the first 15 min was 0,7 % h^{-1}.

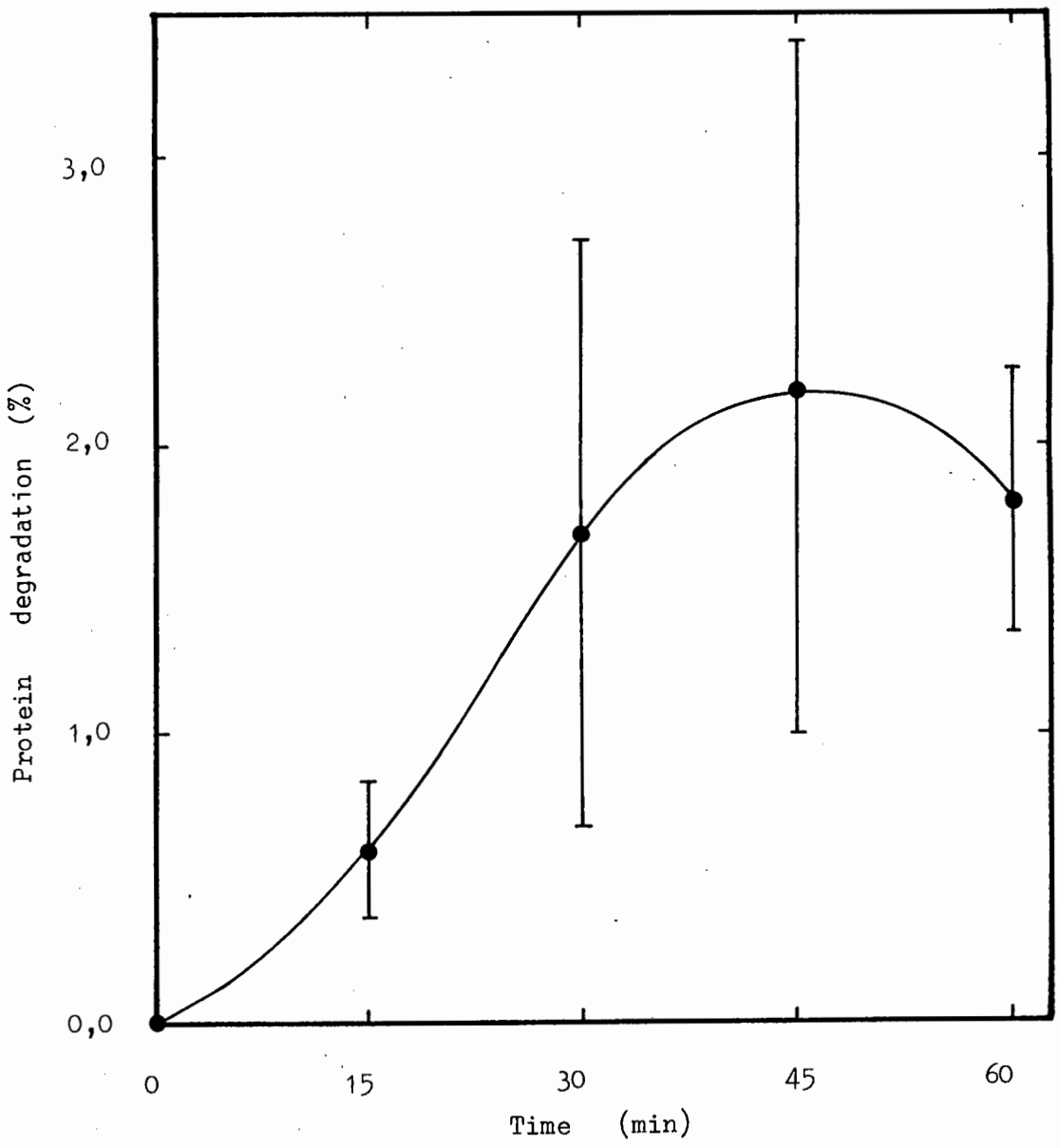


Fig. 2,5. Protein degradation in standing stationary phase cells in the presence of chloramphenicol.

Prelabelled standing stationary phase cells were resuspended in 'spent' medium containing 100 μg ml^{-1} of cml (2.2.3), and protein degradation was determined. These data were the means of 4 independent experiments. The standard deviation is given as bars.

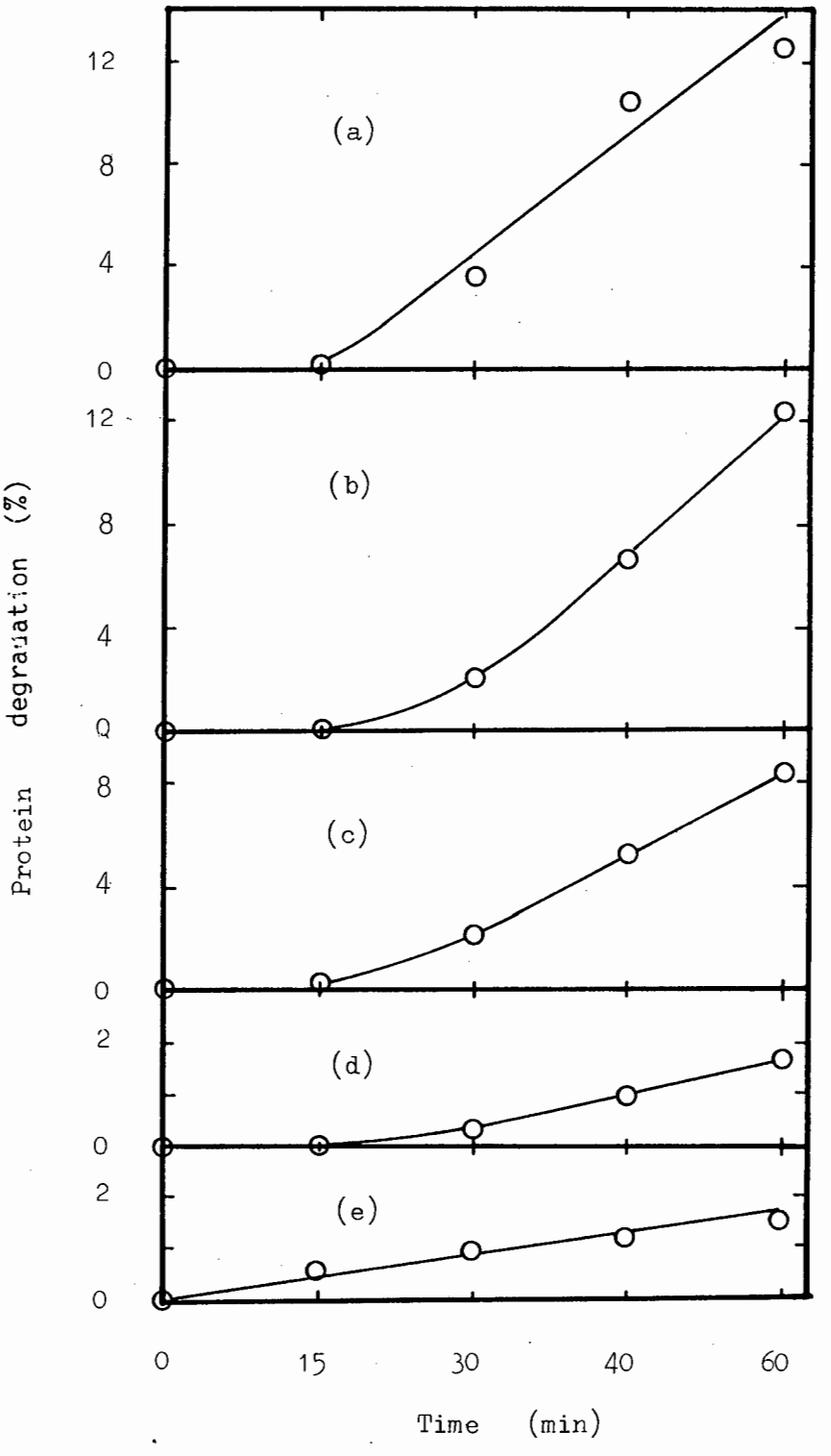


Fig. 2.6. Protein degradation in shaking stationary phase cells.

Shaking stationary phase cells were incubated with radiolabel for 1 h (a); 2 h (b); 6 h (c); 12 h (d); 3 d (e), prior to the measurement of protein turnover. Turnover rates were (in % h^{-1}); (a) 17,8; (b) 20,6; (c) 12,4; (d) 2,5; (e) 1,0; calculated from the linear portions of the graphs.

2.3.2. Protein turnover in Vibrio.

Protein turnover in Vibrio cells was measured under several different conditions (Table 2.1) by measuring the release of radiolabel from prelabelled cells. The rate of turnover in Vibrio cells fell from 1,8 % h^{-1} in exponentially growing cells (which were aerated by shaking exactly as were shaking stationary cells) to 1,0 % h^{-1}. In standing stationary phase cells, the turnover rate was 2.9 % h^{-1}. Both exponentially growing cells treated with chloramphenicol and starved exponential cells treated with chloramphenicol showed higher rates of turnover (6,4 and 7,7 % h^{-1} respectively). However, stationary phase cells were not affected by starvation and the addition of cml in the same manner. Turnover in stationary phase cells increased 3-fold when the cells were resuspended in a growth limiting medium (starved), but on addition of cml to either starving or non-starving cells, the rate of turnover did not change significantly. Manipulation of standing stationary phase cells caused large variations in the data (Figs. 2.4 & 2.5). The rate of turnover in these cells fell from 2,9 % h^{-1} to 0,7 % h^{-1} when the cells were resuspended in a growth-limiting medium, and the addition of cml to standing stationary phase cells caused turnover to fall to 2,1 % h^{-1}.

These turnover rates refer to cells in which the proteins had been extensively labelled (grown for a least two generations in the presence of radiolabel, as with exponential cells, or grown since early exponential phase, in the case of stationary phase cells).

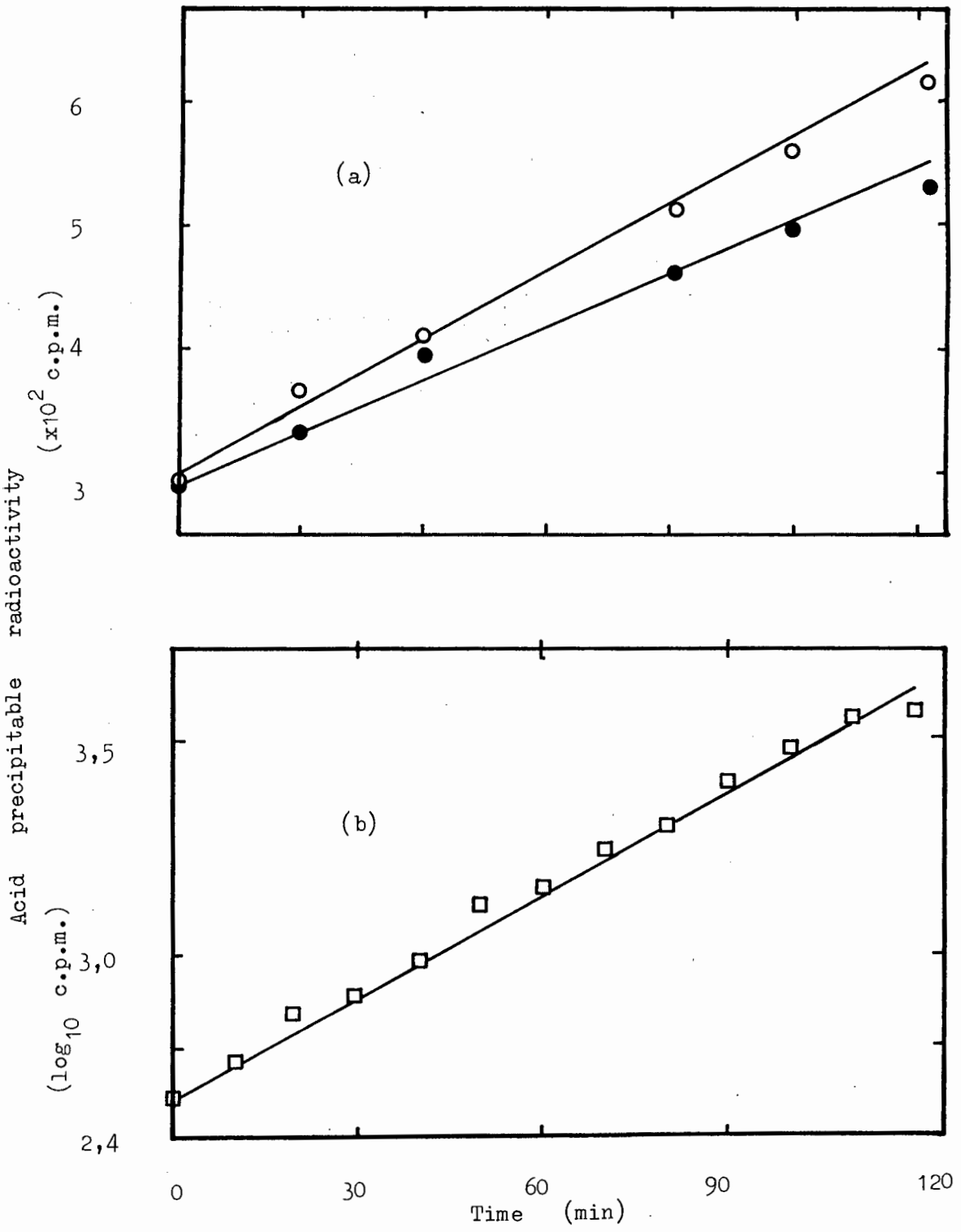


Fig. 2.7. Incorporation of [^{32}P]-orthophosphate into TCA precipitable materials by *Vibrio* cells.

Radiolabel was added to the cultures, and the acid-precipitable radioactivity was determined (2.2.2). (a) Shaking (o), and standing (•) stationary phase cells; (b) exponential phase cells.

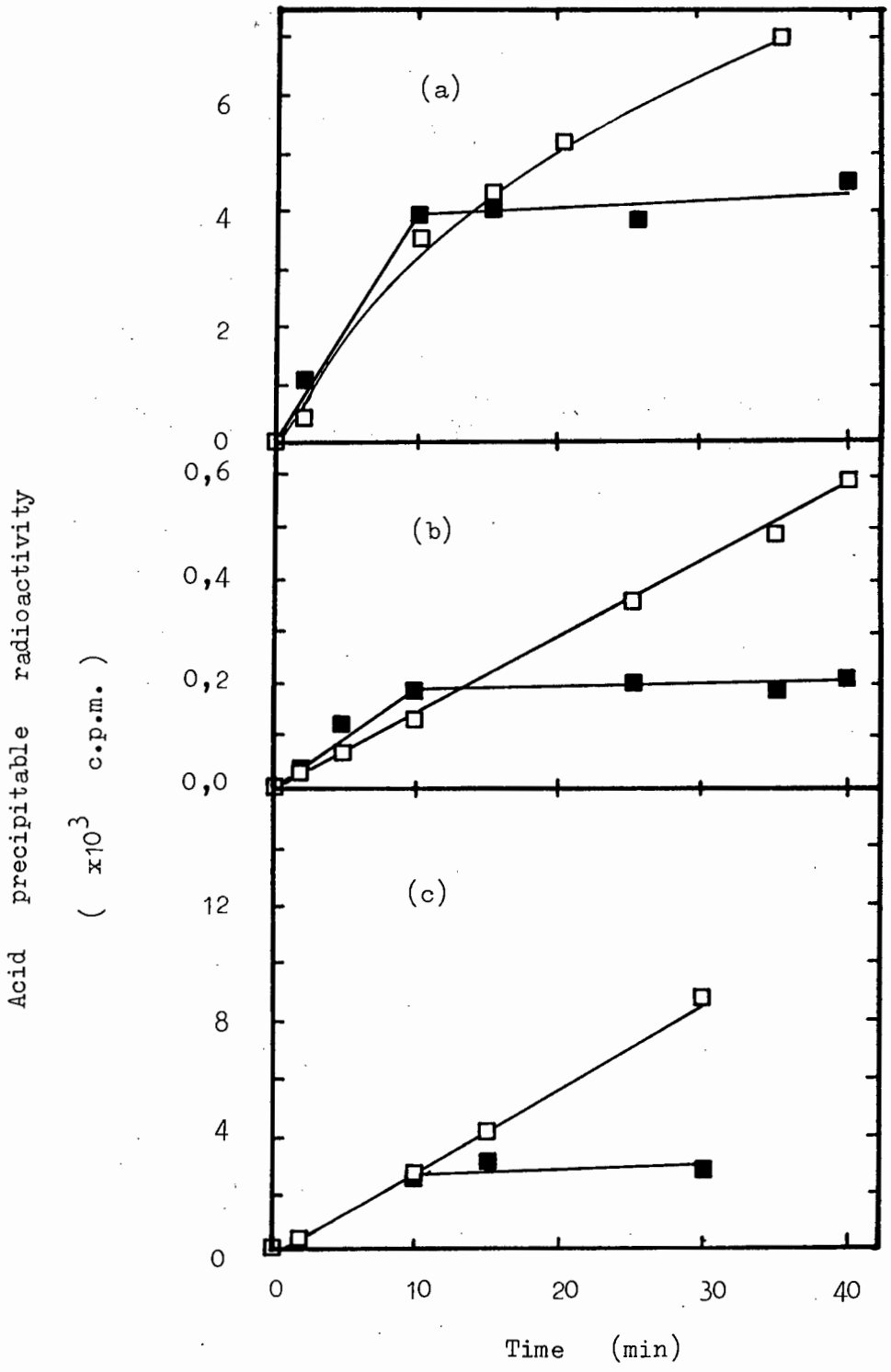


Fig. 2.8. Effect of 10 mM hydroxylamine on macromolecular synthesis in *Vibrio* cells.

Macromolecular synthesis was followed by determining the incorporation of (a) [^{3}H]-uracil; (b) [^{3}H]-adenine and (c) [^{35}S]-methionine in exponential phase *Vibrio* cells. Hydroxylamine (10 mM) was added 10 min after the addition of radiolabel (■), except to parallel control cultures (□).

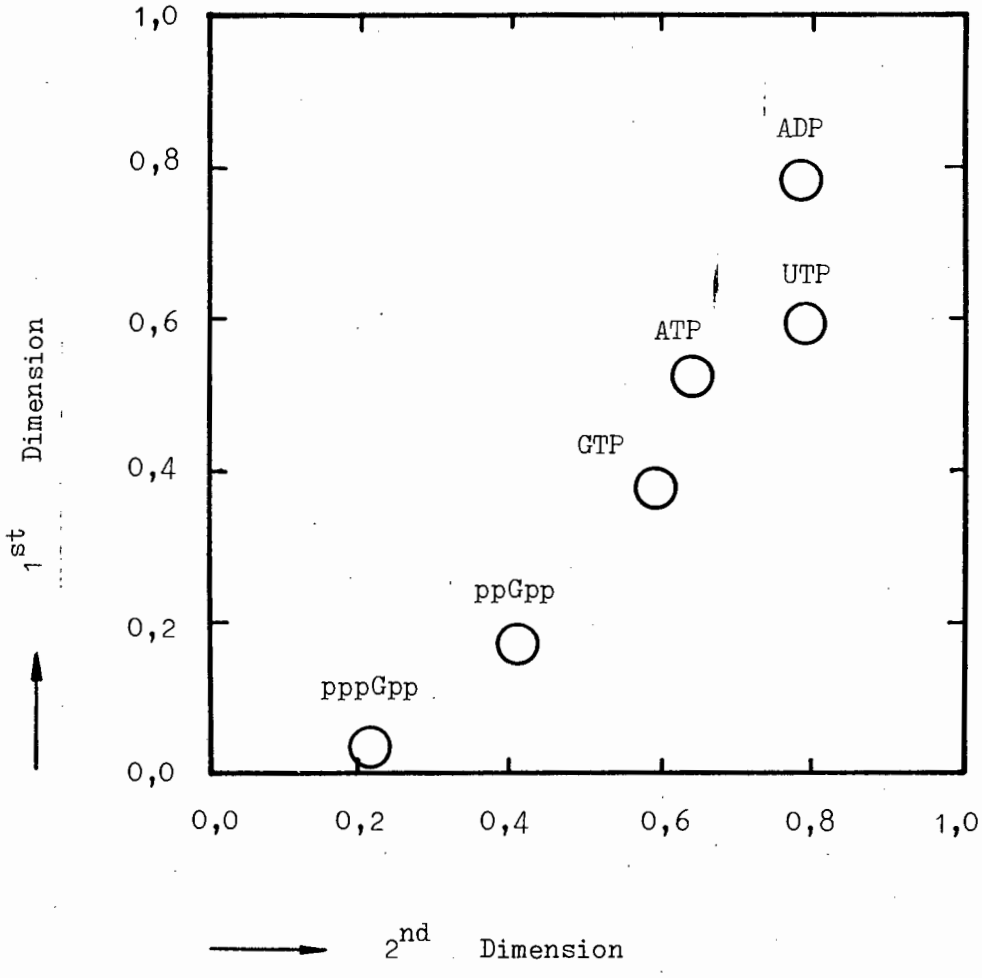


Fig. 2.9. Chromatography of Nucleotide Standards.

Standards (3µl of a 10 mg ml^{-1} solution) were individually chromatographed as described (2.2.4), detected by flourescence in U.V. light and the positions marked. This is a composite diagram of results.

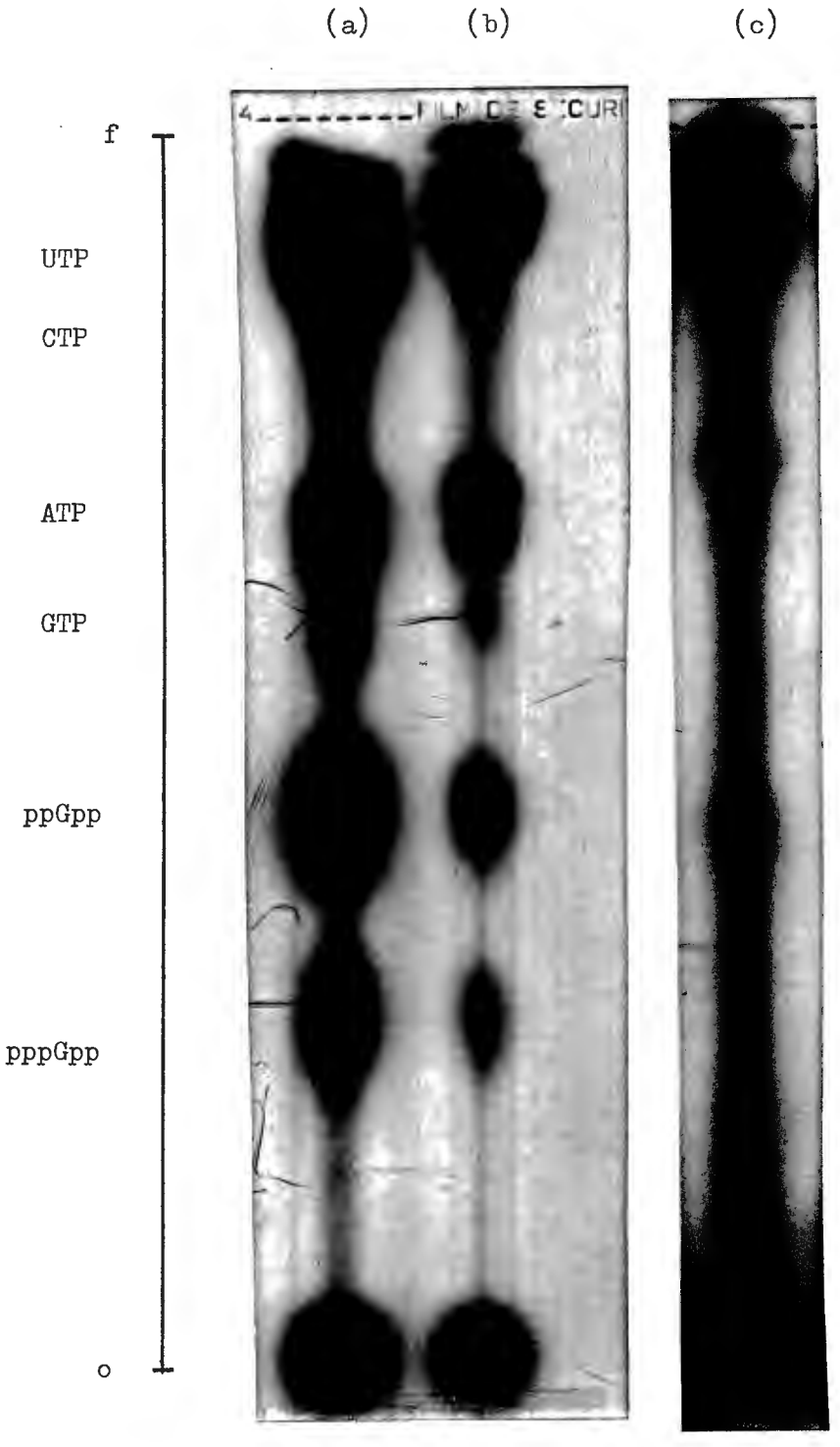


Fig. 2.10. Autoradiographs of one dimensional chromatograms of Vibrio exponential phase nucleotide extract.

Hydroxylamine was used to induce ppGpp synthesis (2.2.4). Standards were run on adjacent tracks with the samples, and their positions marked. An aliquot (3 µl) of an hydroxylamine induced cell extract (a), 1 µl of the same extract (b), 3 µl of an unmanipulated extract (c), the sample was taken prior to the addition of hydroxylamine.

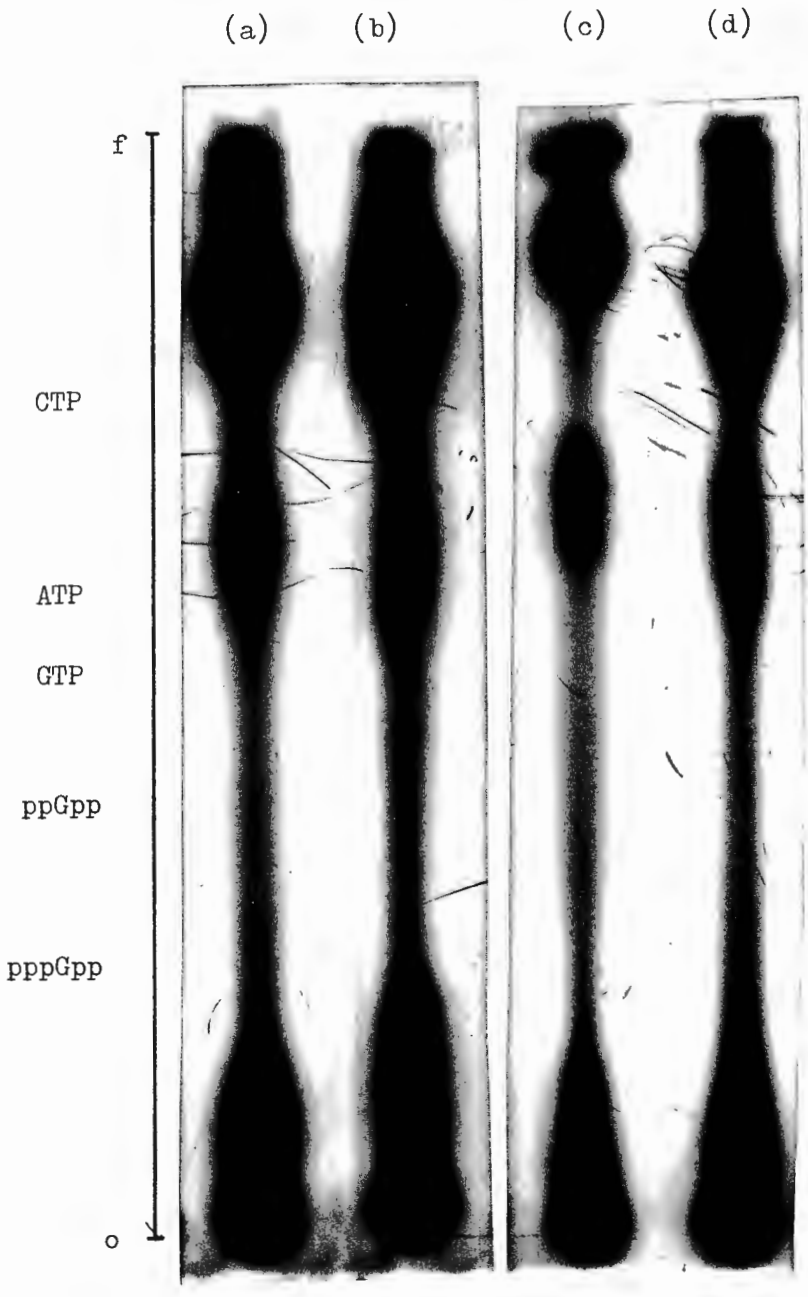


Fig. 2.11. Autoradiographs of one-dimensional chromatograms of stationary phase Vibrio nucleotide extract.

Aliquots (3 µl) of stationary phase extracts were chromatogrphed as described (2.2.4). Shaking stationary phase cell extract, with (b) and without (a) the addition of hydroxylamine. Standing stationary phase cell extract, (d) with and without (c) the addition of hydroxylamine.

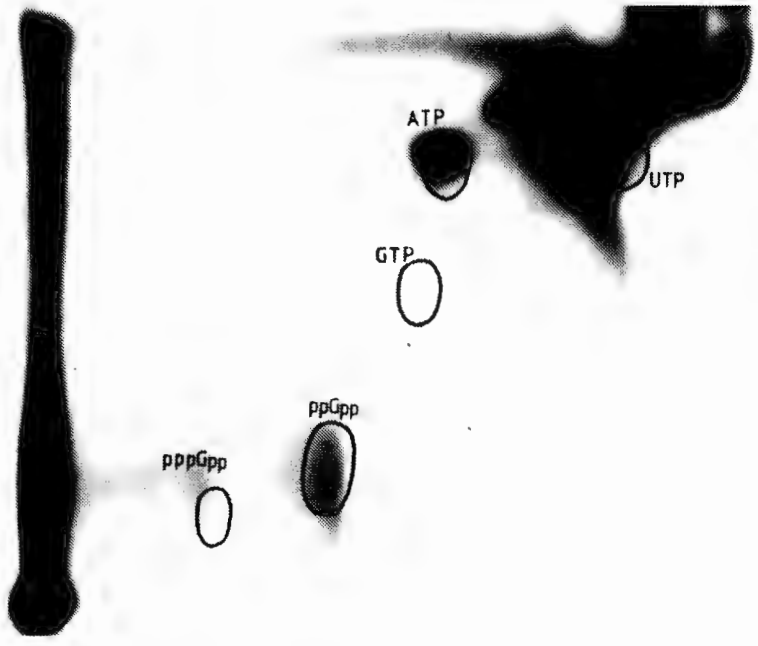


Fig. 2.12. Autoradiograph of a two dimensional chromatogram of exponential phase Vibrio nucleotide extract.

The cells were induced for ppGpp accumulation with hydroxylamine. Standards were run concurrently on a separate thin layer plate and the positions of the standards are depicted in the diagram.

Protein turnover in starving exponential cells showed biphasic kinetics (Fig. 2.3.) with an initial large rate of 14,2 % h^{-1}, which fell after 30 min to 4,2 % h^{-1}.

Deviation in the data between independent experiments was never greater than 8 % (% deviations are given in Table 2.1) except in experiments involving standing stationary phase cells, where the deviation was large (Figs. 2.4 & 2.5). Turnover continued linearly for the time measured (60 min), and control experiments showed that turnover continued linearly for at least 2 h.

Rates of protein turnover were substantially higher in aerated stationary phase cells when the pulse period was reduced from 3 d to 1 h (Fig. 2.6). The rate increased to between 10 and 20 % h^{-1}, with short pulse periods. Consistent results were not obtained with standing stationary phase cells in this type of experiment.

2.3.3. ppGpp in Vibrio.

[^{32}P]-Orthophosphate was taken up by Vibrio cells in broth, as shown by its incorporation into TCA precipitable materials (Fig. 2.7). This means that nucleotide pools in Vibrio must also have been labelled. The rate of incorporation of [^{32}P]-orthophosphate in stationary phase cells is linear and at a much lower rate, approx. 0,7 c.p.m. min^{-1} [calculated from Fig. 2.7 (a)], than that in exponential phase cells, in which the incorporation is exponential [Fig. 2.7 (b)]. This may have been due to the lack of detectable DNA synthesis, and the generally lower rate of

metabolism in stationary phase cells (Robb, 1979). For this reason, stationary phase cells were labelled with [^{32}P] for 24 h before nucleotide pool analysis.

Lund and Kjeldgaard (1972) reported that hydroxylamine induced ppGpp accumulation in E. coli, as well as immediately arresting both protein and RNA synthesis. They suggested that a challenge with hydroxylamine was an experimentally useful way to test bacterial strains for their ability to form ppGpp.

Hydroxylamine (10 mM) arrested both protein and RNA synthesis in exponentially growing Vibrio cells [Fig. 2.8 (a) & (c)]. Similar results were obtained with respect to RNA and protein synthesis in stationary phase cultures. However, although hydroxylamine induced ppGpp synthesis in exponential phase Vibrio cells [Fig. 2.10 (a) & (b)], it did not do so in stationary phase cells [Figs. 2.11 (b) & (d)]. A basal level of ppGpp was detected in exponentially growing Vibrio cells [Fig. 2.10 (c)] but not in stationary phase Vibrio cells [Fig. 2.11. (a) & (c)]. The identification of ppGpp and pppGpp was confirmed by two dimensional chromatography of hydroxylamine induced exponential phase extract (Fig. 2.12). Authentic standards were spotted and co-chromatographed with the sample, then observed under U.V. lighting at 254 nm. The positions of the standards were recorded. A trace amount of GTP was visible in the samples from exponential phase cells run in one dimension [Fig. 2.10 (a) & (b)], but was not visible on the two dimensional chromatogram. GTP was also not visible in stationary phase extracts run in one dimension (Fig. 2.11). This lack of GTP, particularly in shaken stationary phase cells (Fig. 2.11(a)) may explain the pattern of protein synthesis (Fig. 2.1) and the physiological differences observed in this study, in that GTP is required in the formation of the ternary complex during protein synthesis, and for RNA synthesis.

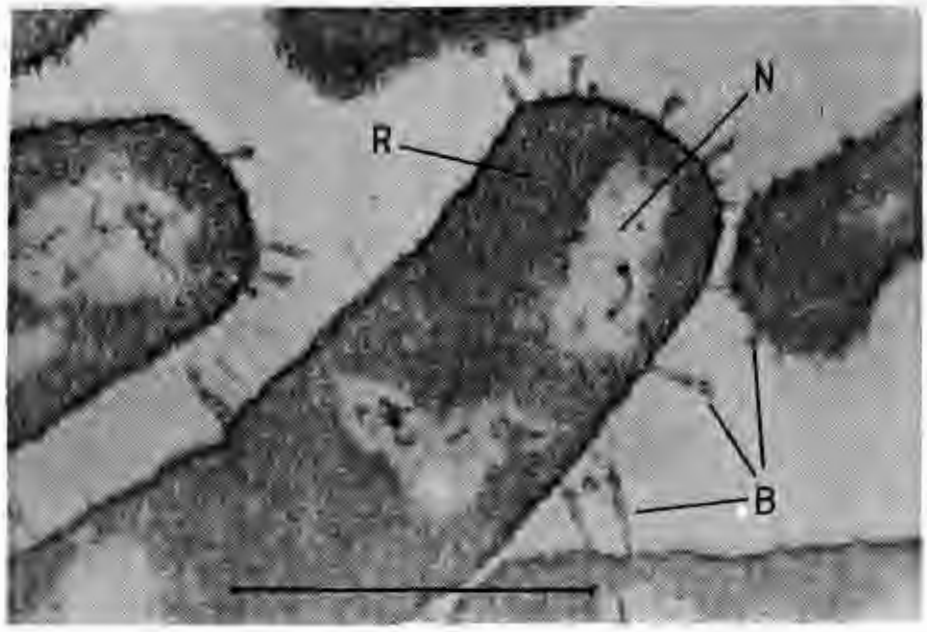


Fig. 2.13. Electron micrograph of thin-sectioned exponential phase *Vibrio* cells.

Thin sections were prepared as described (2.2.5.1) and viewed. A representative micrograph is presented. The presence of blebs on exponential phase cells appeared to be extensive, but not inevitable. The bar represents 1,0 μm; ribonucleoprotein (R); blebs (B) and nuclear material (N).

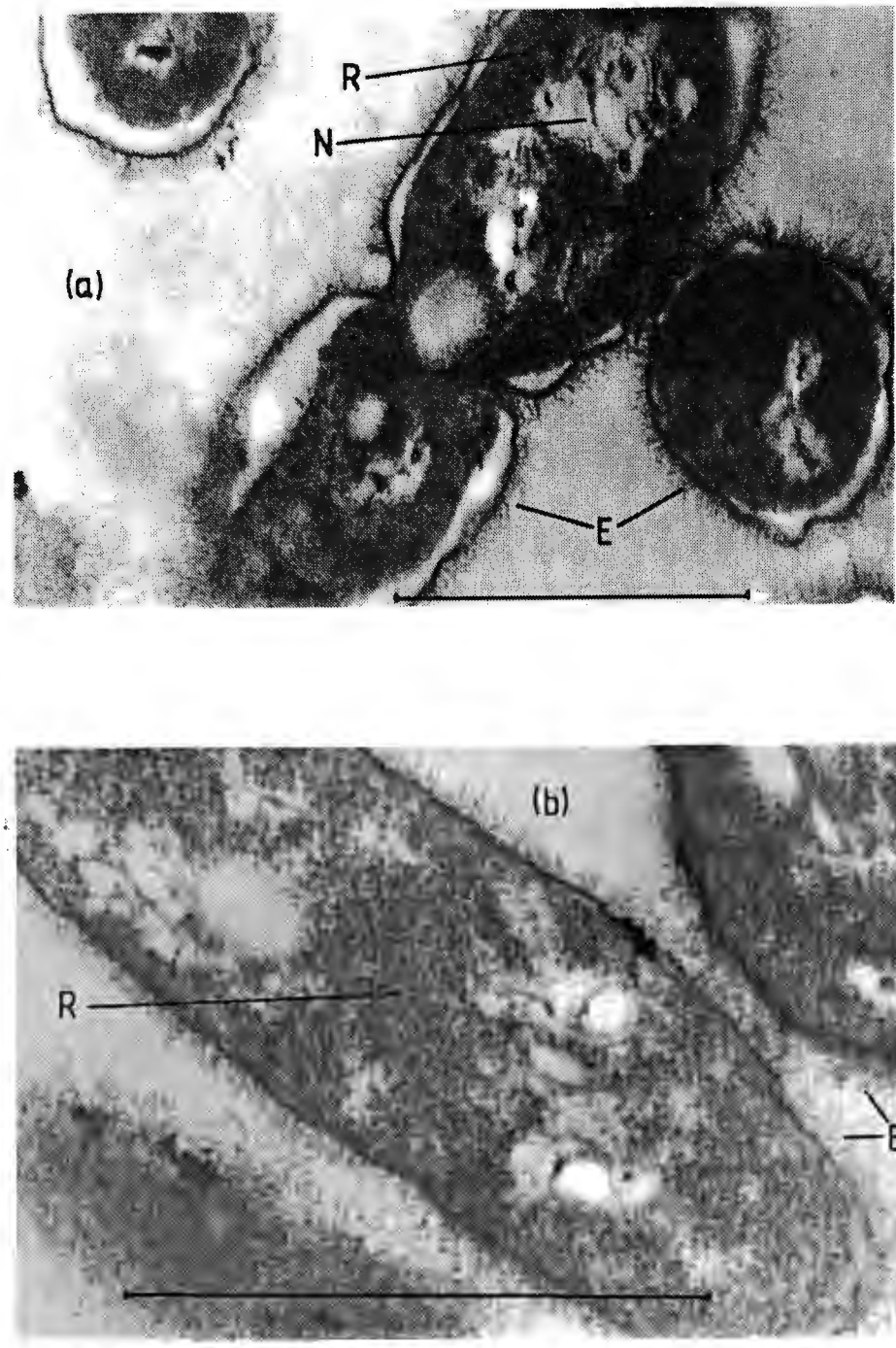


Fig. 2.14. Electron micrographs of thin-sectioned exponential phase *Vibrio* cells.

Exponential phase cells were incubated for one cell doubling in (a) hypertonic and (b) hypotonic medium prior to fixing, thin sectioning and viewing. Representative micrographs are shown. The bars represent 1,0 µm; ribonucleoprotein (R); nuclear material (N) and extracellular material (E).

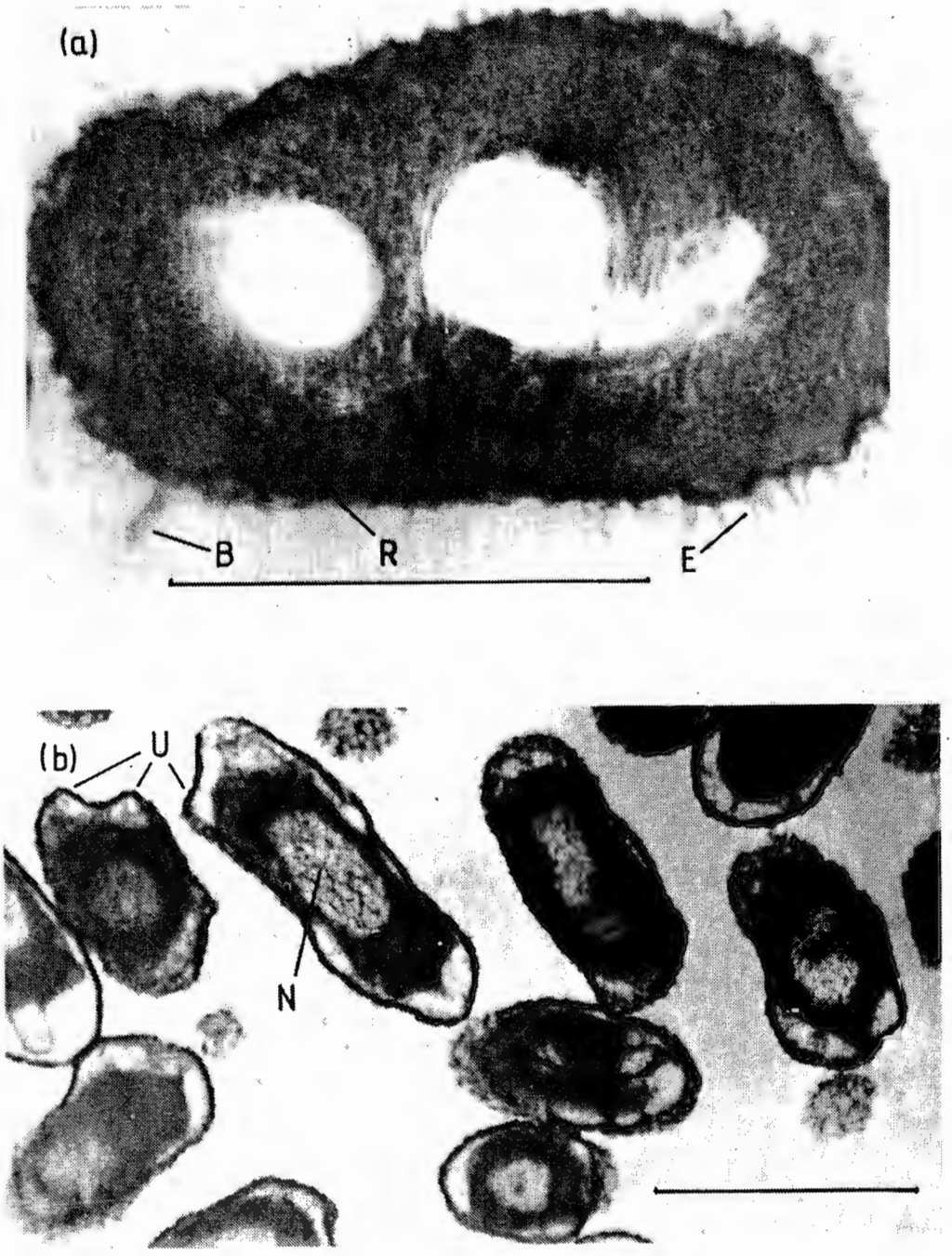


Fig. 2.15. Electron micrographs of thin-sectioned stationary phase *Vibrio* cells.

Thin sections were prepared as described (2.2.5.1) and viewed. Blebs (B) persisted into early (15 h) stationary phase (a), and extracellular polymeric material was evident (E), but neither were present after 3 d of shaking (b), although the cells had undulant walls (U); nuclear material (N). The bars represent 1,0 μm.

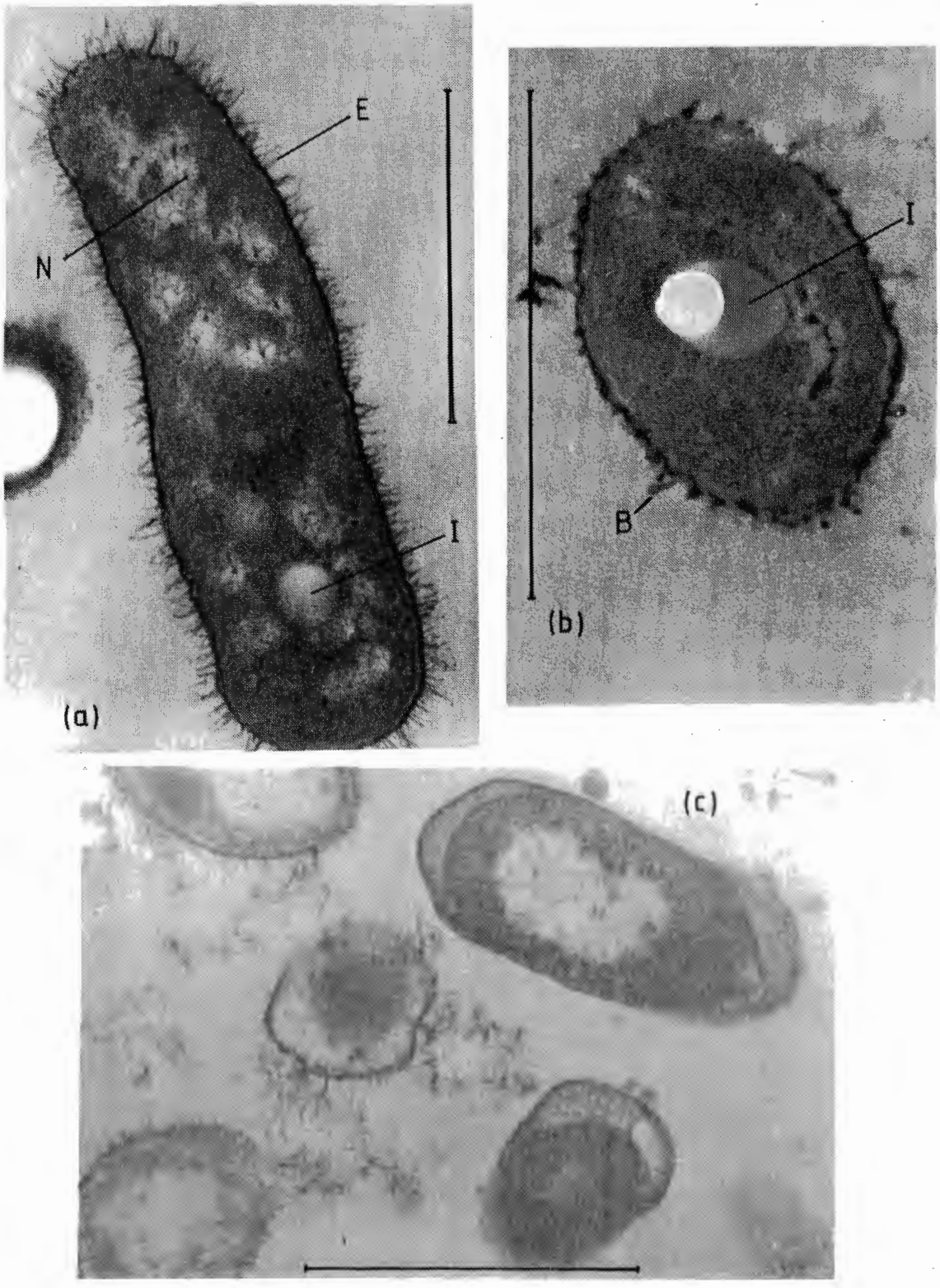


Fig. 2.16. Electron micrographs of thin-sectioned standing stationary phase cells.

Thin sections were prepared as described (2.2.5.1) and viewed. Cell with extracellular polymeric material (E) and electron-light inclusion (I) bodies (a); cell with 'blebs'(B) and inclusion (I) bodies (b); intact cells and an apparently disintegrating cell (c). The bars represent 1,0 µm.

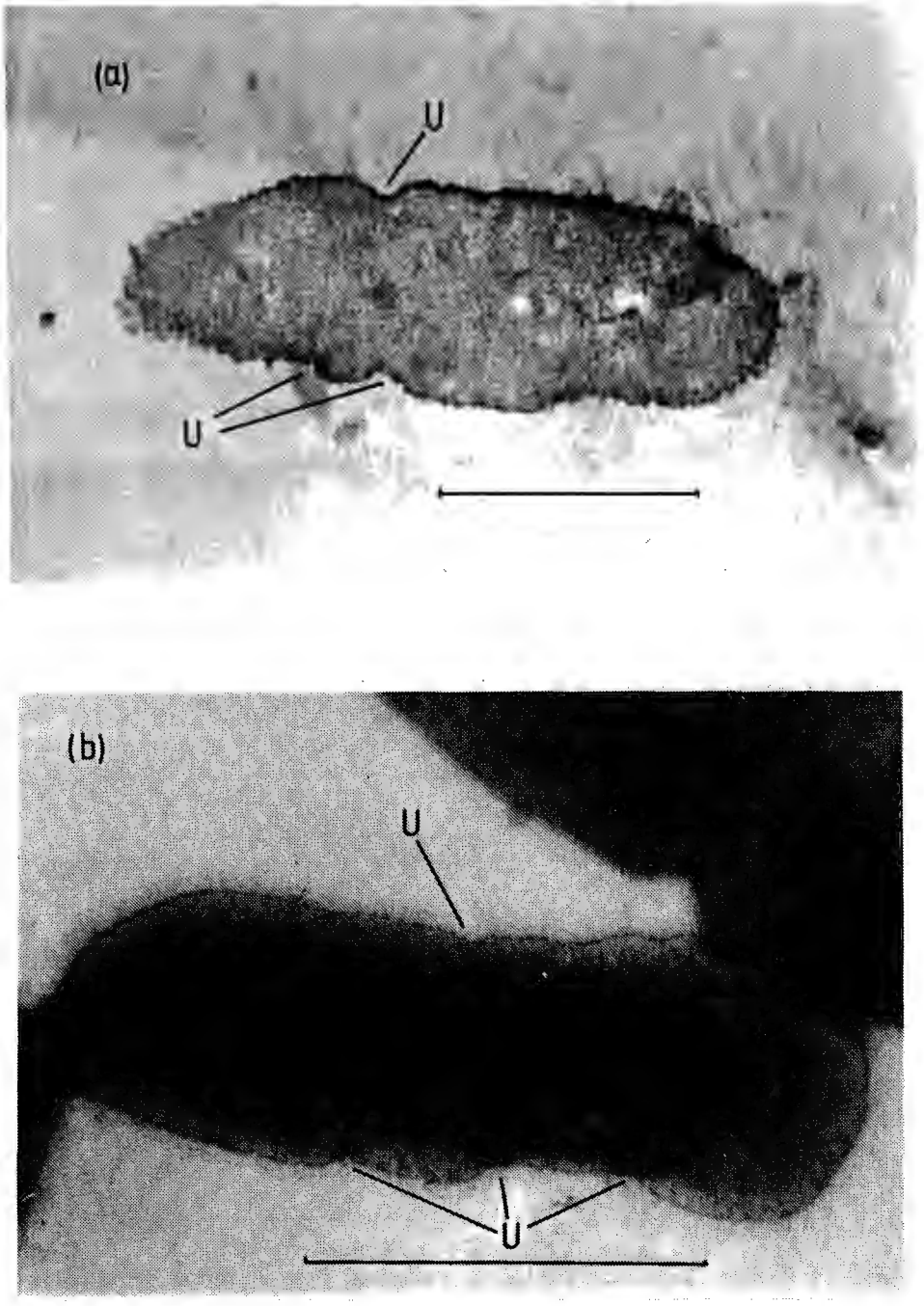


Fig. 2.17. Electron micrographs of shaking stationary phase Vibrio cells.

The irregularly shaped (U) cell wall of some shaking stationary phase cells is shown in a thin-section preparation (a) and a negatively stained preparation (b). The bars represent 1,0 µm.

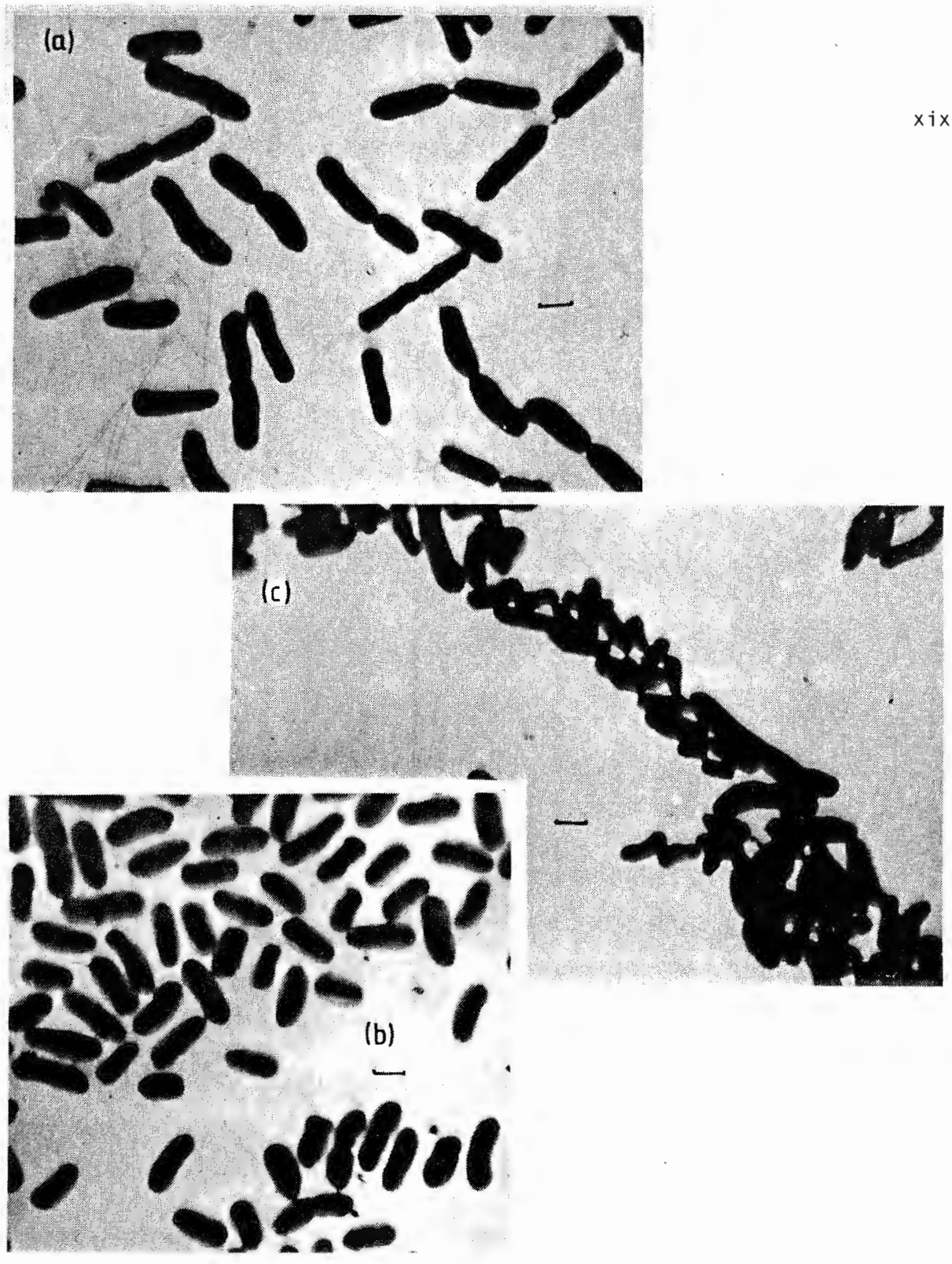


Fig. 2.18. Electron micrographs of negatively-stained Vibrio cells.

Cells were negatively-stained, viewed and extensively photographed. Representative micrographs are given. Exponential phase cells (a), showing the presence of flagella, and marked rod shape, shaking stationary phase cells showing the general absence of flagella and the pronounced "ovality" of the cells (b) and standing stationary phase cells showing the heterogenous nature of cell lengths and clumping of cells (c). The bars represent 1,0 μm.

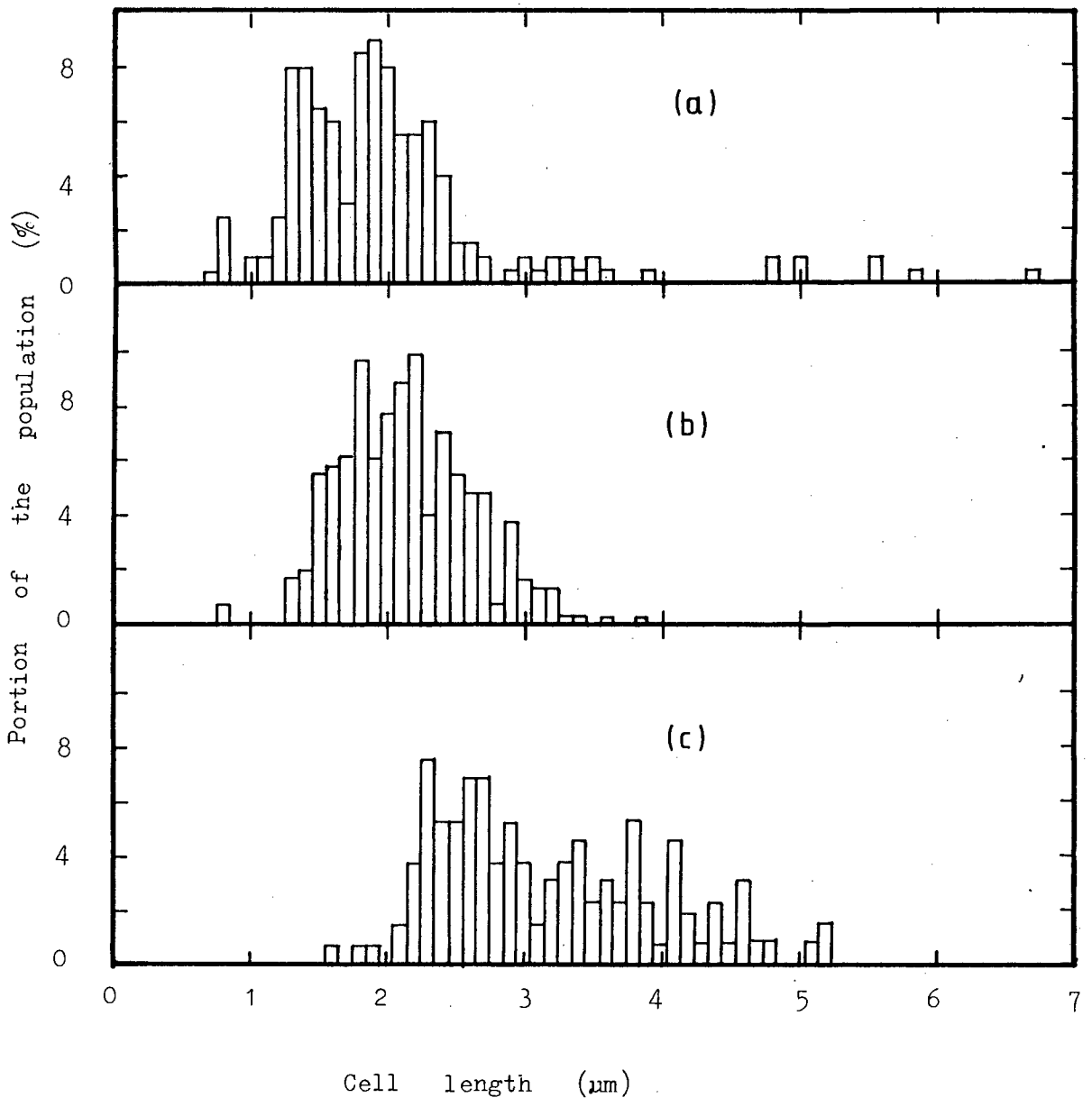


Fig. 2,19. Distribution of cell lengths in *Vibrio* cultures.

Cell lengths were determined by measurement of electron micrographs of negatively-stained (PTA) cells. (a) Standing stationary phase cells; (b) shaking stationary phase cells; and (c) exponential phase cells. For each of the growth phases, samples of cells from several independently grown cultures were mixed, stained and viewed. Data from between 800 and 1000 individuals was used.

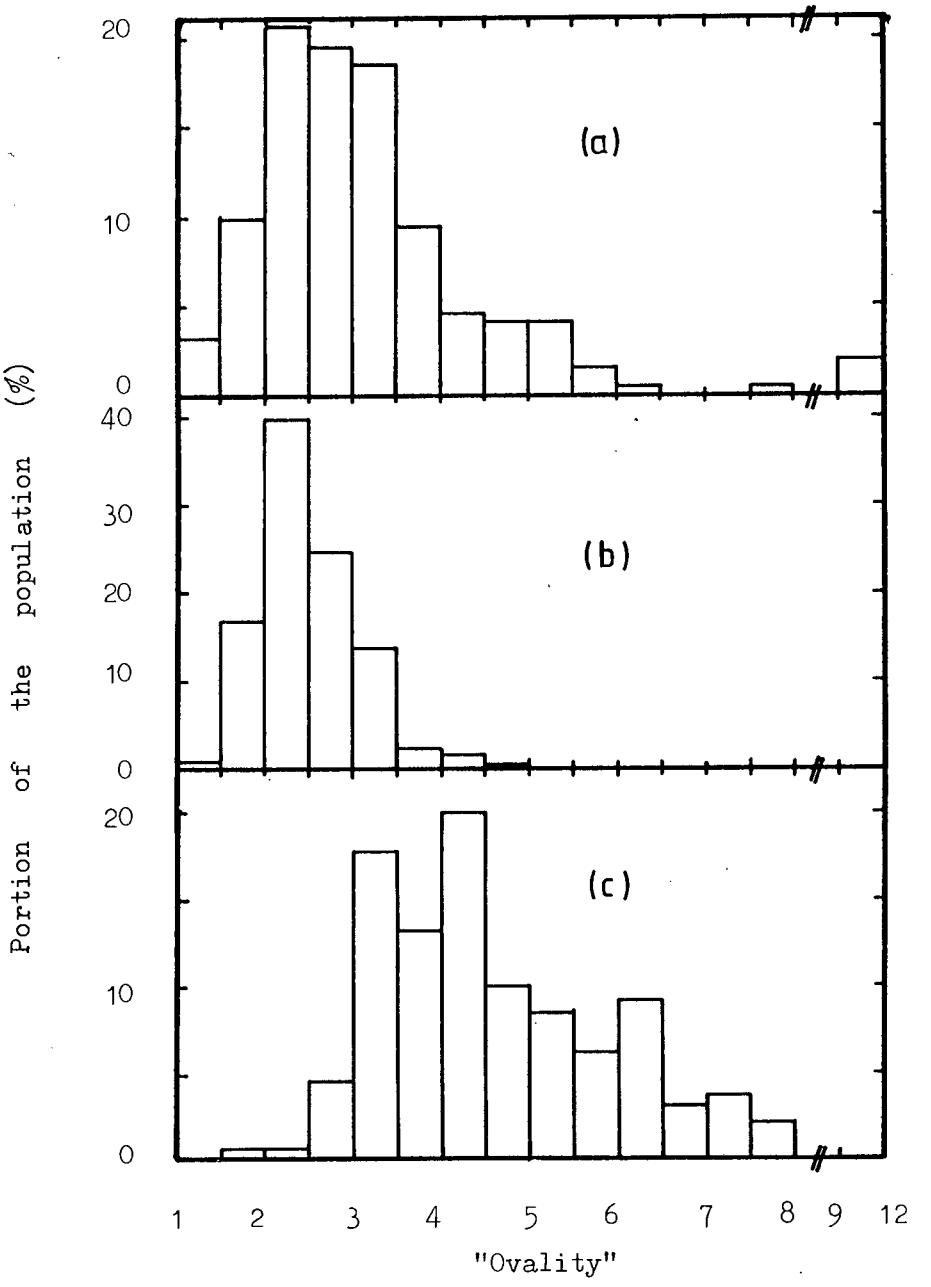


Fig. 2.20. "Ovality" of *Vibrio* cells.

Lengths and widths of *Vibrio* cells were measured from electron micrographs of negatively stained cells, as described (Fig. 2.19). "Ovality" is the ratio of length to width (L/W) of (a) standing stationary phase; (b) shaking stationary phase and (c) exponential phase *Vibrio* cells.

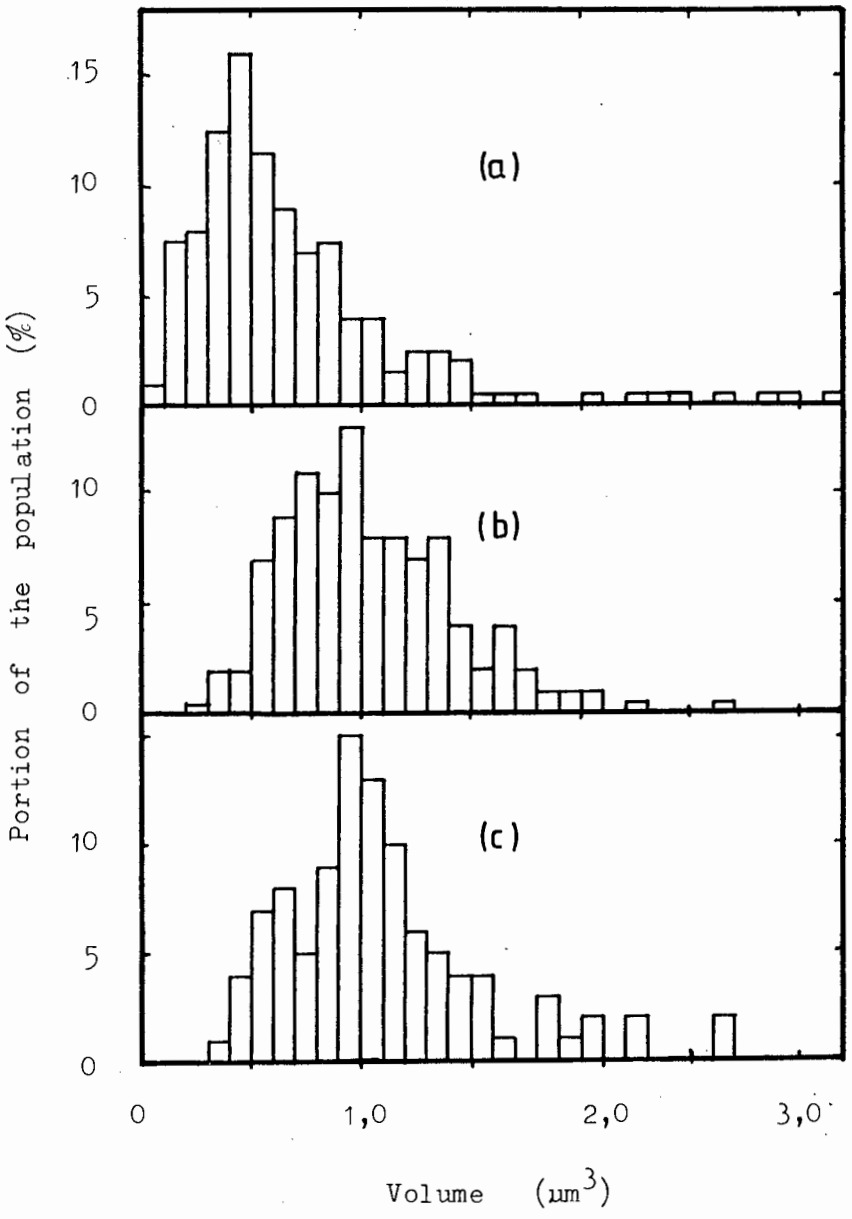


Fig. 2.21. **Volume of Vibrio cells.**

The volume of cells was calculated (2.3.5) from the measurements of length (L) and width (W) of the cells (Figs. 2.19; 2.20) for (a) standing stationary phase cells; (b) shaken stationary phase cells; and (c) exponential phase cells. The average volume of exponential cells was 1,19 ± 0,44 μm^3; of shaken stationary cells, 1,12 ± 0,37 μm^3, and of standing stationary cells, 0,68 ± 0,55 μm^3.

2.3.4. *Vibrio* cell morphology and cell size.

Thin sections of stained *Vibrio* cells in the three growth phases were examined by electron microscopy. *Vibrio* has the typical trilaminar plasma membrane/cell wall and axially disposed nuclear material seen in vibrios (Kennedy *et al.*, 1970). Distinct ribonucleoprotein particles were visible in all phases (Figs. 2.13; 2.15 and 2.16).

Exponential phase cells appeared to have several distinct bodies of nuclear material. A feature of exponential cells was the presence of blebs on the cell wall. Blebs were usually, but not inevitably, observed on exponential phase preparations, and were present (but to a much lesser degree) on early stationary phase cells [Fig. 2.15 (a)]. Blebs were not observed in exponentially grown cells incubated in either hypertonic or hypotonic medium prior to thin sectioning [Fig. 2.14 (a) and (b)].

The presence of electron-light inclusion bodies and extracellular polymeric materials was frequently observed in and on standing stationary phase cells [Fig. 2.16 (a) & (b)] but seldom observed in the other two phases, although extracellular polymers were observed in exponential cells grown in either hyper- or hypotonic media (Fig. 2.14) and in shaking early-stationary phase cultures [Fig. 2.15 (a)].

Curvature of the cells, typically seen among vibrios, was evident in exponentially growing cells, but shaken stationary phase cells were typically coccoid in appearance (Fig. 2.18). The presence of very long

cells was observed in standing but not shaken stationary phase preparations. Shaken stationary phase cells often had undulant cell walls (Figs. 2.15 and 2.17) and appeared plasmolysed. Cells which appeared to be disintegrating were occasionally observed in standing stationary phase preparations [Fig. 2.16 (c)].

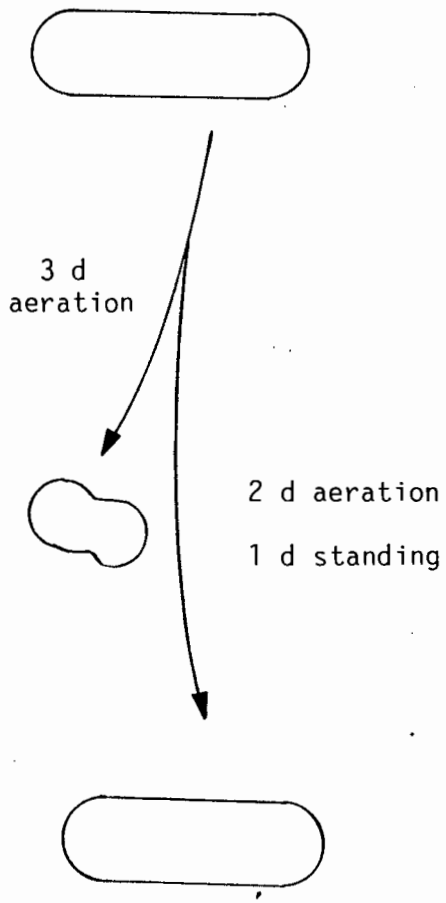
Lengths and widths of Vibrio cells were measured from micrographs of negatively-stained preparations (Fig. 2.18). The preparations were extensively photographed and the measurements compiled (Figs. 2.19; 2.20). The average cell length for exponentially growing cells was 3,16 $\pm$ 0,81 µm, that of shaken stationary phase cells was 2,11 $\pm$ 0,45 µm, and that of standing stationary phase cells was 2,01 $\pm$ 0,87 µm. Cell lengths in exponential phase cultures [Fig. 2.19 (c)] were distributed in the fashion typical of bacteria in balanced growth (Ingraham et al., 1983). 'New born' cells appeared to have a length of approximately 2 um, and the length would appear to have doubled before cell division occurred. Shaken stationary phase cells had a much smaller range of cell lengths [Fig. 2.19 (b)]. Standing stationary phase cells had a large range of cell lengths [Fig. 2.19 (a)], although the majority (over 80 %) of the cells had lengths of between 1,25 and 2,25 µm. Very long (>5 µm) and small (<1 µm) cells together made up approximately 10 % of the population.

With continued shaking, Vibrio cells became coccoid in stationary phase, that is, the 'ovality' (L/W) of the cells decreased markedly in the change from exponential [Fig. 2.20 (c)] to shaken stationary phase [Fig. 2.20 (b)]. Standing stationary phase cells showed a greater range in the ovality of the cells [Fig. 2.20 (a)]. The volume of Vibrio cells

Exponential phase cell

large, motile

able to support Alpha 3a growth



Shaking stationary phase cell

small, non-motile 'sick'

unable to support phage growth

low ATP content per cell

irregular cell shape

Standing stationary phase cell

large, motile 'healthy'

able to support phage growth, more resistant to u.v. and thermal inactivation than shaking stationary phase cells

Fig. 2.22. Model of Vibrio sp.2 stationary phase phenomena.

This diagram depicts the model of Vibrio sp.2 development as proposed by Robb, 1979.

was computed from measurements of length and width ($[4/3.\pi.(W/2)^3]+[(L-W).\pi.(W/2)^2]$) with the assumption that the cells are cylinders with hemispherical ends. The average volume of shaken stationary phase cells was slightly smaller (1,12 $\pm$ 0,37 μm^3) than that of exponentially growing cells (1,19 $\pm$ 0,44 μm^3), and the average volume of shaken stationary phase cells was 57 % smaller, at 0,68 $\pm$ 0,55 μm^3 (Fig. 2.21).

2.4. Discussion.

Robb (1979) proposed a model to explain the stationary phase phenomena observed in Vibrio sp. 2. The model is depicted in Fig. 2.22. In this case, 'healthy' is a comparative term describing the increased resistance to thermal and U.V. inactivation, increased motility and cell size, the increased cellular level of ATP, and the ability to support phage Alpha 3a growth, observed in standing stationary phase cells compared to shaking stationary phase cells. One parameter that apparently does not fit the model is that of protein synthesis. Shaken stationary phase cells incorporate radiolabelled amino-acids into TCA precipitable material at a much higher rate than do standing stationary phase cells (Robb, 1979; Robb et al., 1980; Figs. 2.1 & 2.2). Robb has suggested that this may be due to uncontrolled protein synthesis, with the subsequent futile recycling of amino acids, as the regulation of protein synthesis was prevented in some way by aeration.

RNA synthesis, as measured by the incorporation of radiolabelled uracil into TCA precipitable materials, is on the other hand low in shaken stationary phase cells, but much higher in standing stationary phase

cells [Woods, 1976; Fig. 2.1 (b)]. Bacterial cells exhibiting stringency (1.3.) in response to starvation or very slow growth (1.4.1) have a much lowered rate of RNA synthesis. A characteristic of the stringent response is the production of ppGpp (1.3.). Exponentially growing Vibrio sp. 2 cells have a basal level of ppGpp [Fig. 2.10 (c)] which increases under the stress of hydroxylamine presence [Fig. 2.10 (a) & (b)]. However, the presence of neither ppGpp nor its precursor, pppGpp could be detected in stationary phase cells (Fig. 2.11), despite extensive labelling of the nucleotide pools with [^{32}P] (Fig. 2.7) as shown by the detection of radiolabelled ATP in stationary phase cells (Fig. 2.11). The intracellular level of ppGpp in stringent cells is about 1 mM (Gallant 1979), a level detectable by this system (Lagosky & Chang, 1980).

The most widely used method for the extraction of ppGpp from cells, 1 M formic acid at 0 ^{o}C (Cashel & Gallant, 1969), has been shown to cause the in vitro degradation of this nucleotide (Lagosky & Chang, 1978). For this reason, the extraction of phosphorylated nucleotides from Vibrio sp. 2 was performed by the method of Lagosky & Chang, 1980. This method relies on the enzymatic disruption of the cells at neutral pH (2.2.4).

It has been found that in certain circumstances, relA^{+} E. coli cells are able to exhibit stringency without the accumulation of ppGpp (Spadaro et al., 1981; 1.3.2). Furthermore, highly phosphorylated nucleotides other than ppGpp and pppGpp have been shown to be associated with energy source regulatory phenomena (Gallant et al., 1976; 1.3.2) and sporulation in B. subtilis. As ppGpp and pppGpp were not detectable in

stationary phase Vibrio cells, it is probable that these nucleotides are not involved in the stationary phase phenomena observed in Vibrio sp. 2.

In this study of Vibrio, protein synthesis has been measured by the incorporation of exogenous [^{14}C]-leucine (Fig. 2.1.) or [^{35}S]-methionine (Fig. 2.2). Factors such as the size of amino acid pools and protein turnover (Nath & Kock, 1971) could possibly affect this measurement. Protein turnover in exponentially growing Vibrio sp. 2 cells (Table 2.1) was found to be similar to that reported for other bacterial cells, as discussed in 1.5.1, that is about 2 % h^{-1} during active growth, which value doubled upon starvation (transfer to growth limiting medium). However, further treatments, such as the blocking of protein synthesis by the addition of cml (Table 2.1) increased the rate of protein turnover, and starvation in the presence of chloramphenicol increased turnover further.

The addition of protein synthesis inhibitors, such as chloramphenicol or tetracycline, to cells starved for amino acids caused a fall in protein catabolism (Mandelstam, 1960; Goldberg & St. John, 1976). There was a concomitant rapid fall in the level of ppGpp in the cells in these circumstances, and this was believed to be involved in the regulation of the turnover rate (St. John et al., 1978). This does not necessarily apply to carbon/energy source downshifts, as the mechanism of inducing the stringent response differs from that involving amino acid starvation (Gallant, 1979).

Initially, the rate of turnover in starving Vibrio sp. 2 cells (previously in exponential growth) was very high (ca. 14 % h^{-1}) which

fell to about 4 % h^{-1}, and remained at this level for at least 2 h (Fig. 2.3). The existence of two kinetically distinct reactions in protein turnover has been shown in E. coli (Pine, 1970). In that case, however the initial high turnover (of 5 % of the radiolabelled amino acid turning over within 45 s) was associated with maturative cleavage of proteins following assembly, and was followed by a slower rate (2.5-3,0 % h^{-1}) of true protein turnover. The initial high rate of protein turnover in starving Vibrio sp. 2 was probably not associated with maturative cleavage because the high rate was sustained for c. 30 min.

Protein turnover measured in shaking stationary phase Vibrio sp. 2 cells was lower than that in exponentially growing cells, and it increased upon starvation. Further treatment, such as the addition of chloramphenicol to either starving or non-starving cells did not change the turnover rate significantly (Table 2.1) from that measured without manipulation.

Overall protein degradation in bacteria has been shown to increase with the slowing of bacterial growth (Goldberg & St. John, 1976) until in stationary phase, it resembles that seen during starvation. The measurement of protein turnover by following the appearance of acid soluble radiolabel from previously labelled protein does not distinguish between a large turnover rate of a small number of proteins, from the low turnover of a large number of proteins (1.5.1). The small turnover rate in shaking stationary phase cells was measured after the extensive radiolabelling of cellular proteins - the cells had been grown since exponential phase, in the presence of radiolabel.

When the extent of labelling was reduced, by reducing the time of pulse labelling, very large rates of protein turnover were measured in shaking stationary phase cells (Fig. 2.6). This suggests that the high level of protein synthesis measured in these cells is balanced, at least partially, by the high level of protein turnover. It has been shown that proteins synthesised during stationary phase, or starvation, were more unstable than the original cell protein (1.5.1), and this appears to be the case with Vibrio sp. 2.

Protein turnover in standing stationary phase cells was higher than in shaking stationary phase cells (Table 2.1). However, upon experimental manipulation of the cultures (required to determine turnover during starvation and/or with the addition of chloramphenicol), the variability in the results was large (Figs. 2.4 & 2.5). Experiments designed to determine protein turnover in standing stationary phase cells with short periods of pulse labelling yielded no meaningful data (2.3.2) despite carefully repeated attempts. Robb (1979) reported that protein synthesis in standing stationary phase cells responded very quickly (within a few minutes) to aeration, which may be a feature of the adaptability of stationary phase cells. Slowly growing E. coli cells increased the specific rate of protein synthesis by a factor of 7 within 2 min after enrichment (Koch & Deppe,1971). Hence, experimental manipulations such as removal of excess radiolabel by washing, might have caused changes in the rate of protein synthesis, affecting the measurement of radiolabel release from protein. This may have made the measurement of protein turnover in these cells meaningless, after extensive experimental manipulation.

The results of light microscopic studies of Vibrio sp. 2 have been included in the model (Fig.2.22). Analysis using thin-sectioned and negatively-stained Vibrio sp. 2 preparations by electron microscopy revealed more detailed changes that occurred during Vibrio sp. 2 development.

A feature of exponentially growing Vibrio cells was the presence of 'blebs' (Fig. 2.13), that is, radial projections of the cell wall/outer membrane, about 0,2 μm in length. Blebbing appeared to be extensive in exponentially growing Vibrio cells, although not all of the cells in a particular preparation had blebs. The extent of blebbing appeared to decrease in early stationary phase (10 - 20 h cultures) until after 3 d of aeration, blebs were not present (Fig. 2.15). Standing stationary phase cells occasionally had blebs [Fig. 2.16 (b)].

In a survey of the fine structure of 20 pseudomonades and more than 15 achromobacters (Vibrio sp. 2 was initially classified as an Achromobacter strain; 1.1.2), Wiebe & Chapman (1968) found that some strains characteristically produced evaginations (blebs) of the cell wall. Bleb length varied from strain to strain and also within a single strain. Those strains regularly produced blebs under growth and fixation conditions identical to those of the strains which did not form blebs. In addition, other strains produced blebs only in specific physiological and nutritional conditions. In that study, the appearance of blebs was neither enhanced nor completely suppressed by adjusting the ionic concentration of the buffers used during fixing. Incubation of exponentially growing Vibrio cells in hyper- or hypotonic medium prior to fixing and thin-sectioning the cells (2.2.5.1) abolished blebbing,

and resulted in the production of extra cellular polymeric material (Fig. 2.14).

Wiebe & Chapman (1968) suggested that a possible role blebs might have played in the ecology of those strains was in the physical attachment of cells to particle surfaces. Such a role has been proposed by Marshall (1985) for the tubular extensions of the cell wall observed in Vibrio DW1. Those tubular extensions appeared to be extensions of the cell wall, approximately 0,3 μm in length. In the case of Vibrio DW1, the extensions appeared on the cells as the cell volumes decreased and their adhesion to surfaces became enhanced.

With the continued aeration of Vibrio sp. 2 cells into stationary phase there was a tendency to plasmolysis, decreased cell length and irregularity of cell shape (Figs. 2.15; 2.17). Furthermore, cell length distribution became smaller as aerated cultures aged from exponential phase to 3 d old stationary ones [Fig. 2.19 (c) & (b)]. However, shortening of cell length was partially compensated for by an increase in cell diameter so that the volume of 3 d old cells was on average < 10 % smaller than that of exponentially growing cells (2.3.5). This suggests that lateral wall formation was checked more than transverse wall formation in shaking stationary phase cells, giving shorter, wider cells, whereas in standing stationary phase cells the opposite may have occurred, yielding narrower, longer cells.

Baker & Park (1975) reported that when grown in batch culture, Vibrio NCTC 4716 showed a distinct sequence of morphological forms. While the cells occurred as almost straight rods during exponential phase, the predominant form in the decline phase was spherical. The spheres were not viable, and survival of the species depended on the few rod forms that remained.

Kock (1884) first reported the vibrioid morphology, and since then other investigators have cited the 'round body' forms peculiar to vibrios (reviewed by Kennedy et al., 1970). Vibrio sp. 2 did not show round body formation, however standing stationary phase cells had a much greater range in cell length, ovality and volume (Figs. 2.19; 2.20 & 2.21) than did shaking stationary phase cells, and the average volume was about 57 % of the volume of shaking stationary phase cells. Standing cultures became very viscous due to the production of extracellular polymeric material, and gradually the cultures separated into two phases, a bottom layer in which the cells were suspended in a viscous, sticky matrix and the upper less viscous layer in which the number of cells gradually fell. After about 2 months, all the cells were concentrated within the lower viscous phase, below a clear upper layer. Stationary phase cultures were able to support Alpha 3a growth for at least 20 d (Woods, 1976). The presence of extracellular polymeric material is evident in Fig. 2.16 (a) & (b). Shaken stationary phase cells did not produce extracellular polymeric materials even after extensive incubation.

CHAPTER THREE.

PROTEOLYTIC ACTIVITY OF VIBRIO CELL-FREE EXTRACTS.

3.1. Introduction.

The elevated levels of protein synthesis measured in shaking stationary phase cells is balanced, at least in part, by the high level of turnover of newly synthesised protein (2.4.). Robb (1979) and Robb et al., (1980) suggested that the relatively high levels of protein synthesis in 'unhealthy' shaken stationary phase cells may be due to non-specific and erroneous transcription, similar to that reported in relaxed mutants (Hall & Gallant, 1972; Travers, 1976). This might result in a high proportion of abnormal proteins in shaken stationary phase cells. Bacterial and animal cells rapidly degrade proteins with abnormal conformations (Goldberg & Dice, 1974). It has been suggested that some bacterial cells may contain two degradative systems with different physiological roles, one that is present in all cells, with the function of removing abnormal proteins, and an adaptive system that is activated on starvation (review, Goldberg & St. John, 1976). Shaken stationary phase cells may have specific or enhanced proteolytic systems, in order to deal with a high level of abnormal protein. For this reason, the proteolytic activity in Vibrio sp. 2 cell-free extracts was investigated using the foreign proteins [^{14}C-me]globin and [^{125}I]-insulin.

[^{14}C-me]globin was rapidly degraded by the same proteolytic system that hydrolysed analogue-containing or puromycyl polypeptides in reticulocyte lysates (Etlinger & Goldberg, 1977). In E. coli, the degradative system responsible for the energy-dependent degradation of abnormal proteins was similar to, if not the same as that system responsible for the hydrolysis of [^{14}C-me]globin and the disappearance of the nonsense fragment of beta-galactosidase, 545 (Murakami et al., 1979; 1.5.2). Those proteases in E. coli that degrade proteins such as casein and apohaemoglobin had little or no activity against small proteins such as insulin or glucagon (Goldberg et al., 1980; 1.5.2) and vice-versa. For these reasons, both [^{14}C-me]globin and [^{125}I]-insulin were used in this study.

Protein degradation in E. coli is energy dependent (Kowit & Goldberg, 1977) and in cell-free extracts, it is stimulated by ATP (Murakami et al., 1979). This ATP-stimulated proteolytic system appears to be responsible for the energy dependent degradation of abnormal proteins. Amongst the many differences between shaken and standing stationary phase Vibrio cells is the difference in cellular ATP concentration (Robb, 1980;), hence the effect of ATP on proteolytic activity in Vibrio sp. 2 cell-free extracts was also investigated.

3.2. Materials and methods.

3.2.1. Media and growth conditions.

The media and growth conditions were as described (2.2.1) however, larger volumes of culture were routinely grown. Exponential cultures were usually grown in 5 l volumes, distributed equally among 5 two-litre round flat bottomed flasks on a Gallenkamp rotary shaker at 150 o.p.m. Each 1 l volume of the broth was seeded (1:100) from an overnight Vibrio sp. 2 culture and grown in parallel. Exponential phase cultures were harvested at (A_{600}) between 0,8 and 0,9. Shaken stationary phase cultures were grown likewise, but incubated for 3 d. To produce standing stationary phase cultures 500 ml volumes of cultures that had been shaken for 3 d were transferred to one-litre Schott blue-capped bottles and incubated aerobically without shaking for 1 d. Standing stationary phase cells produced in this way were able to support phage Alpha 3a growth. For the production of starved cells, cells from exponential and both types of stationary phases were harvested and washed by centrifugation (10 000 g for 10 minutes), resuspended in a pre-warmed growth limiting minimal medium [Tris/HCl, 100 mM, pH 7.6; NaCl, 0,4 M; $(NH_4)_2SO_4$, 7,6 mM; Mg Cl_2 0,4 mM; sodium citrate, 1.6 mM; glucose 0.03 % (w/v)] and incubated.

3.2.2. Preparation of cell-free extracts.

Exponential, stationary, and starved cultures were harvested and washed twice by centrifugation at 10 000 g for 10 min. The pellet of cells was frozen with liquid nitrogen and lysed by one of three methods, the lysozyme-EDTA method, the Yeda press method, or by grinding with alumina.

3.2.2.1. The lysozyme-EDTA lysis method.

Cells were lysed by a modification of the method of Wickner et al., (1972). After thawing the pellet, the cells were resuspended in TEAS buffer (50 mM triethanolamine buffer (pH 8.0) containing 10% (w/v) sucrose) and 100 ug lysozyme ml^{-1} was added (2 ml buffer per g wet mass of cells) and incubated on ice for 30 min. KCl (0,5 M) and 2-mercaptoethanol (2-ME) (10 mM) were added, the lysed suspension was centrifuged at 30 000 g for 30 min at 4 ^{o}C, and the supernatant (S30) was dialysed against TMKD buffer.

3.2.2.2. Lysis using the Yeda press.

After thawing the pellet, the cells were resuspended in TEAS buffer (2 ml per g wet mass of cells) and passed through a Yeda press (Lamon Instrumentation Co. Israel) at 10,3 M Pa, twice. The lysed suspension was centrifuged as above (3.2.2.1).

3.2.2.3. Lysis by grinding with alumina.

This was performed as described by Hughes et al., (1971). Alumina, (washed and dried at 100 °C, then stored at 4 °C) was added to the thawed pellet of cells, mixed to a powder like consistency, and ground with a pre-chilled mortar and pestle until the powder turned to a clay like consistency (less than 5 min). TMKD buffer was added (2 ml per g wet mass of cells) and the alumina, unbroken cells and cell debris removed by centrifugation, as above. The whole procedure was performed at 4 °C.

3.2.3. Determination of protein concentrations.

Protein concentrations were determined by the method of Lowry et al., (1951), or, if there was membrane material present in the samples, by a modification of the method of Markwell et al., (1978). Bovine serum albumin was used as the standard.

3.2.4. Preparation of radiolabelled substrates.

Apohaemoglobin (globin) was radiolabelled by reductive methylation (Rice & Means, 1971; Moore & Crichton, 1973), using [^{14}C]-formaldehyde. Globin was produced from haemoglobin (bovine, Sigma) by the method of Yonetani (1967) or Asakura (1978). [^{125}I]-Insulin was prepared

according to the method of Kirschner & Goldberg, (1981). [^{125}I]-Insulin [Amersham; 0,1 µg (750 uCi ug^{-1}) was mixed with 1,25 mg of non-radioactive insulin (dissolved in 10 mM NaOH), yielding a specific activity of 8 x 10^6 c.p.m. (mg insulin)$^{-1}$. The specific activity of [^{14}C-me]globin was between 1 and 4 x 10^6 c.p.m. (mg protein)$^{-1}$. Non-enzymatic release of radiolabel from these substrates was low (< 0,5 c.p.m. min^{-1}) compared to enzymatic release (never < 5 c.p.m. min^{-1}).

3.2.5. Protein degradation.

The degradation of labelled substrates by cell-free extracts was measured by a modification of the method of Murakami et al., (1979) at 30 °C by incubating 100 µl extract (at pH 8,0 (3.2.2.1;3.2.2.3) or pH 7,6 (93.2.2.2)) with 10 µg of [^{14}C]-radiolabelled substrate or 5 µg of [^{125}I]-insulin (approximately 10^4 c.p.m.) in the presence or absence of nucleotides or inhibitors, all adjusted to pH 8,0 as appropriate. Samples (50 µl) were removed at timed intervals, mixed with 50 µl BSA (30 mg ml^{-1}) as a carrier and diluted into 100 µl cold TCA (10% w/v). After 30 min the samples were centrifuged in an Eppendorf microfuge model 5412 for 1 min and the acid-soluble radioactivity released from the substrates determined. For most of the experiments, the final volume of the reaction mixture was 250 µl from which three or four samples were taken to give time courses of the reactions. In experiments determining pH optima or the effect of inhibitors, the volumes were reduced by half, and two samples (at 0 and 60 min) were taken.

3.2.6. Determination of pH optima.

The optimal pH for proteolytic activity of the extracts was determined as above (3.2.5). Tris/HCl saline buffers, (200 mM) were used to obtain the desired pH above neutrality. Tris/acetate saline buffers (200 mM) were used for pH values of 7,0 and below.

3.2.7. Protease inhibitors.

The effects of the following protease inhibitors were determined; EDTA, o-phenanthroline, p-hydroxymercuribenzoate (pHMB) and phenylmethyl-sulphonyl fluoride (PMSF). An appropriate dilution of the inhibitor (10 µl) was mixed with the cell-free extract (50 µl) for 5 min prior to the addition of labelled substrate (65 µl). PMSF was dissolved in dimethyl sulphoxide immediately prior to use, due to its reported instability (James, 1978). Control experiments indicated that dimethyl sulphoxide had a negligible effect on the proteolytic activity of the cell extracts.

3.2.8. Preparation of subcellular fractions.

Two different methods of preparing subcellular fractions were used. Cytoplasmic and periplasmic extracts were prepared by a modification of the method of Kaback (1971). Cells were harvested, washed by centrifugation, resuspended in TES buffer with 100 µg ml^{-1} lysozyme added, and then incubated on ice for 1 h. The supernatant obtained

after centrifugation at 16 000 g for 10 min was the periplasmic fraction. Sphaeroplast formation in Vibrio sp. 2 was judged by phase contrast microscopy. The pellet was resuspended in TSMMKD buffer and incubated on ice for 30 min, frozen in liquid nitrogen, and then thawed at 10 °C. The supernatant collected after centrifugation at 40 000 g for 45 min was the cytoplasmic fraction. The fractions were dialysed for 12 h at 4 °C against TMKD buffer before determination of the proteolytic activities. The second method, a modification of the method of Nossal & Heppel (1966), was used to produce osmotic fluid ('periplasmic' fraction) and differential centrifugation was used to produce membrane, ribosomal and cytoplasmic enriched fractions (Swamy & Goldberg, 1982). The whole process was performed at 4 °C. Vibrio sp. 2 cells were harvested, washed once by centrifugation, and resuspended in 33 mM Tris (pH 7.6) at 20 ml per g wet mass of cells. An equal volume of 40 % sucrose, Tris/HCl (33mM, pH 7,6) was slowly added with stirring. Sufficient 100 mM EDTA (sodium salt; pH 7,6) was added to give a final concentration of 10^{-4} M, then stirred at room temperature (24 °C) for 10 min. The cells were pelleted by centrifugation (27 000 g for 10 min at 4 °C), drained, rapidly dispersed in ice cold $MgCl_2$ solution (5 x 10^{-4} M; 40 ml per g wet mass of cells), 2-ME added (10 mM final concn.) and the suspension stirred in an ice-bath for 10 min. The osmotic shock fluid was the supernatant after centrifugation (27 000 g; 10 min) which was reduced by ultrafiltration to 1/50 of the original culture volume. The pellet of cells was frozen in liquid nitrogen, thawed and TMM buffer added (5 ml per g wet mass of cells). The cells were lysed by passage twice through a YEDA press at 10,3 MPa. Unbroken cells were removed by centrifugation and the lysate centrifuged at 30 000 g for 30 minutes. The pellet, (P30 representing the membrane fraction) was washed and

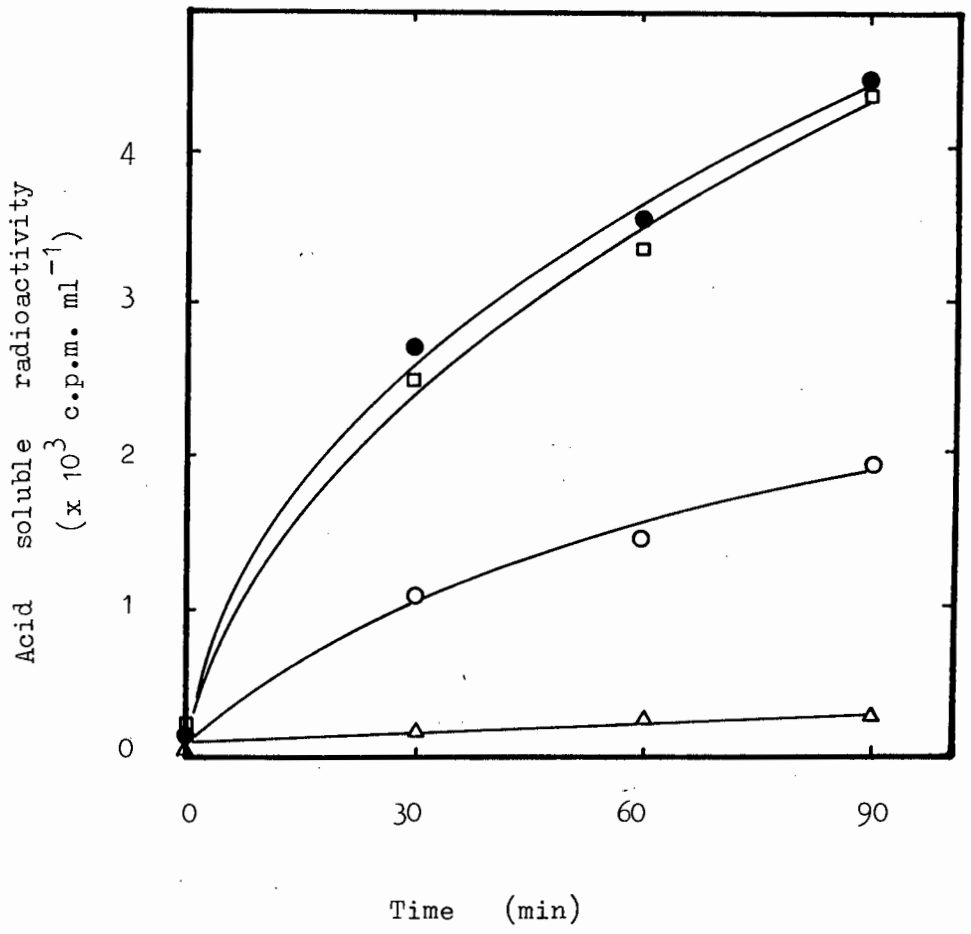


Fig. 3.1. Time course of the release of radioactivity from [^{14}C-me]globin by *Vibrio* cell-free extracts.

The degradation of [^{14}C-me]globin by (□) exponential; (○) shaken stationary; and (●) standing stationary phase cell-free extracts was determined as given (3.2.5). Non-enzymatic release of radioactivity is given (△). Protein concentrations (mg ml^{-1}) in the extracts were, exponential, 22,2 ± 2,8; shaken stationary, 11,3 ± 1,5; standing stationary, 21,7 ± 1,4.

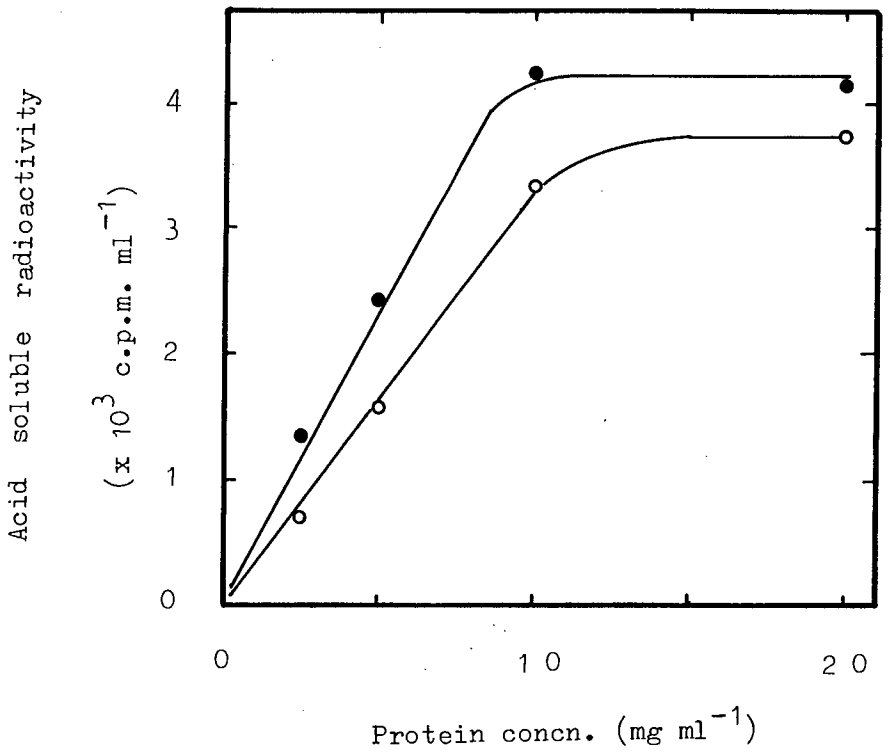


Fig. 3.2. **Effect of the concentration of the exponential Vibrio cell-free extract (S30) on the degradation of [14C-me]globin.**

Proteolytic activity against [^{14}C-me]globin was determined (●) with and (○) without the addition of 3 mM ATP. The extract was prepared by the lysozyme-EDTA method(3.2.2.1), diluted appropriately, and assayed. The assay volume was 250 µl, and 100 µl of the extract was used per assay.

resuspended in TMM buffer at 1/50 of the original culture volume using a pre-chilled Braun homogeniser. The supernatant was centrifuged again at 100 000 g for 2 h. The pellet (P100, represented the ribosomal fraction) was washed and resuspended in TMM in 1/50 of the original culture volume, and homogenised in a pre-chilled Braun homogeniser. The supernatant (S100, represented the cytoplasmic fraction) was concentrated by ultrafiltration to 1/50 of the original cell volume. All of the preparations were distributed into 1,5 ml aliquots and kept frozen at -70 °C until used, but not for longer than 1 week.

3.3 Results.

3.3.1. Proteolytic activity of cell extracts.

The protein concentrations of the dialysed cell-free extracts from exponential and standing stationary phase cells were similar, but the equivalent extracts from the shaken stationary phase cells were routinely half that of other extracts (Fig. 3.1), using either the lysozyme-EDTA or YEDA press methods for lysis, and although the rate of [^{14}C-me]globin degradation by shaken stationary phase cell extracts appears to be lower than that of the other two extracts (Fig. 3.1),when adjusted for protein concentration, the rates of all three extracts are similar. The time course of the release of radiolabel by cell-free extracts indicated a steady rate of degradation for at least 90 min. Generally experiments were performed over 60 min. The amount of substrate did not appear to be limiting, at least for extract concentrations below 10 mg ml^{-1} (1,0 mg protein per assay, see Fig. 3.2) at which concentration subsequent experiments were performed. Control experiments showed that at most 15 % of the available substrate was used in any experiment, although pronase E (from

Table 3.1. Specific activities of the dialysed cell-free extracts.

The cell-free extracts (3.2.2.1) were each diluted to 10 mg ml^{-1} and the activities determined (3.2.5) without the addition of ATP. The activities shown are the means of 4 experiments, ± SD. The specific activity of the [^{14}C-me]globin used was 1,73 x 10^5 c.p.m. mg^{-1} and that of the [^{125}I]-insulin used was 1,3 x 10^5 c.p.m. mg^{-1}.

	Specific activity [µg protein hydrolysed min^{-1} (mg protein)$^{-1}$]	
	against globin	against insulin
Exponential phase	0,57 ± 0,02	0,85 ± 0,02
Shaking stationary phase	0,68 ± 0,08	0,72 ± 0,07
Standing stationary phase	0,62 ± 0,03	0,81 ± 0,03

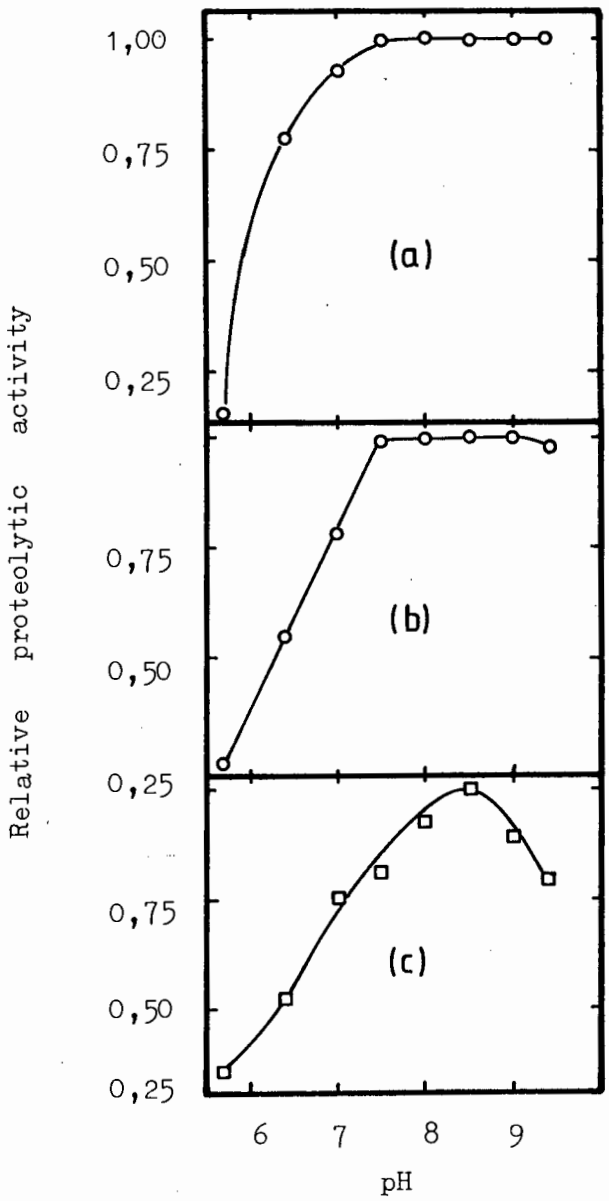


Fig. 3.3. Effect of pH on the degradation of [^{14}C-me]globin by Vibrio cell-free extracts.

Proteolytic activities were determined without the addition of ATP, as given in the text. The results are from one of three experiments, all giving similar results. Activities are given relative to the highest activity obtained. (a) Shaking stationary phase extract (b) standing stationary phase extract (c) exponential phase extract.

Streptomyces griseus, Sigma) at 10 µg ml^{-1}, degraded approximately 80 % of the substrate in 60 min. Control experiments were performed on each batch of substrate prepared. The proteolytic activities of the extracts against [^{14}C-me]globin and [^{125}I]-insulin were determined at the same protein concentrations of cell-free extract and were shown to be similar for the three phases without the addition of ATP (Table 3.1). The activity of the shaken stationary phase cells against [^{14}C-me]globin was between 10 and 20 % higher than that of the other extracts, but the activity against [^{125}I]-insulin was about 15 % less. Although other substrates, [^{14}C-me]casein and [^{35}S]-Vibrio extract (produced by growing Vibrio in the presence of [^{35}S]-methionine, then extracting the protein using TCA precipitation) were used, they were found to give similar results to those obtained using [^{14}C-me]globin, and [^{14}C-me]globin was used preferentially. The use of lysozyme-produced cell extracts for proteolytic determinations has been questioned, due to proteolytic activity of egg white lysozyme (Oliver & Stadtman, 1983) however, this activity concerns the 'auto α' complementation assay (see 1.5.2) and in that study there was only trace proteolytic activity against larger substrates, haemoglobin, casein and albumin. Incubation of [^{14}C-me]globin with lysozyme for 24 h was found not to release radiolabel above the rate of non-enzymatic release (that is, < 0,5 c.p.m. min^{-1}, Fig. 3.1).

3.3.2. Effect of pH on proteolytic activities.

The proteolytic activities of the stationary phase extracts had a broad

Table 3.2. Effect of inhibitors on the degradation of [^{14}C-me]globin by *Vibrio* cell extracts.

Proteolytic activities were determined (3.2.5) without the addition of ATP. The values given are the average of five independent experiments and the amount of variation was < 8 % from the mean.

Inhibitor	Concn. (mM)	Decrease in activity (%) in: Exponential phase	Shaken Stationary phase	Standing Stationary phase
EDTA	10	94	80	84
	5	91	79	84
	1	0	54	25
o-phenanthroline	0,5	51	76	66
PMSF	5	80	83	65
PHMB	5	35	69	67
	1	5	70	66
	0,15	0	72	73

Table 3.3. Subcellular localisation of proteolytic activities in *Vibrio* cells.

Periplasmic and cytoplasmic cell-free extracts were prepared by the method of Kaback (1971) as given (3.2.8) from exponential and both types of stationary phase cells and assayed for proteolytic activity against [^{14}C-me]globin with and without the addition of ATP (3 mM). The data from one of four independent experiments are given. The results from all the experiments were within 7 % of the values given.

Cell-free extract	Activity (c.p.m.)				
	-ATP	Activity as % of total	+ATP	Activity as % of total	Stimulation %
Periplasmic					
Exponential	237	9	349	11	47
Shaken stationary	430	17	523	17	22
Standing stationary	670	23	769	25	14
Cytoplasmic					
Exponential	2396	91	2785	89	16
Shaken stationary	2175	83	2516	83	16
Standing stationary	2261	78	2311	75	2

Table 3.4. Protein distribution in Vibrio subcellular fractions.

Protein concentrations of the subcellular fractions, prepared by the method of Nossal & Hepple (1966) as given (3.2.8), were determined using a modified Lowry procedure (appendix B), and expressed as a percentage of the total protein in that preparation. Values given are the means, and the variation from the mean was < 4 %.

	Relative protein concentration (%)		
		Shaking	Standing
Subcellar fraction	Exponential phase	stationary phase	stationary phase
Periplasmic	12	7	4
Membrane	78	84	84
Ribosomal	2	2	6
Soluble cytoplasmic	8	6	6

Table 3.5. Subcellular distribution of [^{14}C-me]globin degrading activity in *Vibrio* cell extracts.

Subcellular fractions were prepared by the method of Nossal & Hepple (1966) as given (3.2.8). Activities were determined (3.2.5) with and without the addition of 3 mM ATP, and expressed as a percentage of the total globin degraded by each extract. Data from at least 4 independent experiments were used, and the variation was < 7 %, except in the case of the ribosomal fractions (P 100), where the activity was low and up to 50 % variation was found.

Subcellular fraction	Exponential phase		Shaking stationary phase		Standing stationary phase	
	-ATP	+ATP	-ATP	+ATP	-ATP	+ATP
Periplasmic	25	32	23	23	53	11
Membrane	34	28	40	42	39	45
Ribosomal	9	10	11	10	9	14
Soluble cytoplasmic	32	29	25	25	5	30

Table 3.6. Subcellular distribution of [^{125}I]-insulin degrading activity in *Vibrio* cell extracts.

Subcellular fractions were prepared by the method of Nossal and Hepple (1966) as given (3.2.8). Activities are expressed as a percentage of the total insulin degraded by all the fractions of each extract. Data from six independent experiments was used, and the variation was < 8 %, except in the case of the ribosomal fractions (P 100), where the variation was much greater.

	Proteolytic activity (%)		
Subcellular fraction	Exponential phase	Shaking stationary phase	Standing stationary phase
Periplasmic	66	25	9
Membrane	20	35	39
Ribosomal	1	18	5
Soluble Cytoplasmic	13	22	47

Table 3.7. Effect of inhibitors on the proteolytic activity of exponential phase Vibrio subcellular fractions.

Proteolytic activities against both [^{14}C-me]globin and [^{125}I]-insulin in subcellular fractions were determined in the presence of inhibitors (3.2.7) without the addition of ATP. The decrease in activity is expressed relative to the original activity in that fraction. The values given were the average of three independent experiments, which varied < 9 % from the mean.

Subcellular Fraction	Decrease in activity (%) against [^{125}I]-insulin		Decrease in activity (%) against [^{14}C-me] globin	
	EDTA (5 mM)	PMSF (5 mM)	EDTA (5 mM)	PMSF (5 mM)
Periplasmic	4	24	38	0
Membrane	61	62	62	41
Ribosomal	9	0	49	32
Soluble Cytoplasmic	44	46	0	0

Table 3.8. Effect of pre-incubation on the degradation of [^{14}C-me]globin by extracts from exponential and stationary phase *Vibrio* cells.

Dialysed cell extracts (3.2.2.1) were preincubated without ATP at 30 °C for 30 min before the addition of substrate with or without ATP (3 mM). The results are expressed relative to the dialysed extract assayed without pre-incubation or the addition of ATP. The values given are the means of six independent experiments and the variation was < 10 % from the mean except for the preincubated exponential phase extract (Fig. 3.6).

	Dialysed extract, not preincubated		Dialysed extract, preincubated	
Cell-free extract	-ATP	+ATP	-ATP	+ATP
Exponential	1	1,76	1,07	3,70
Shaken stationary	1	1,73	1,29	1,63
Standing stationary	1	1,76	1,33	1,50

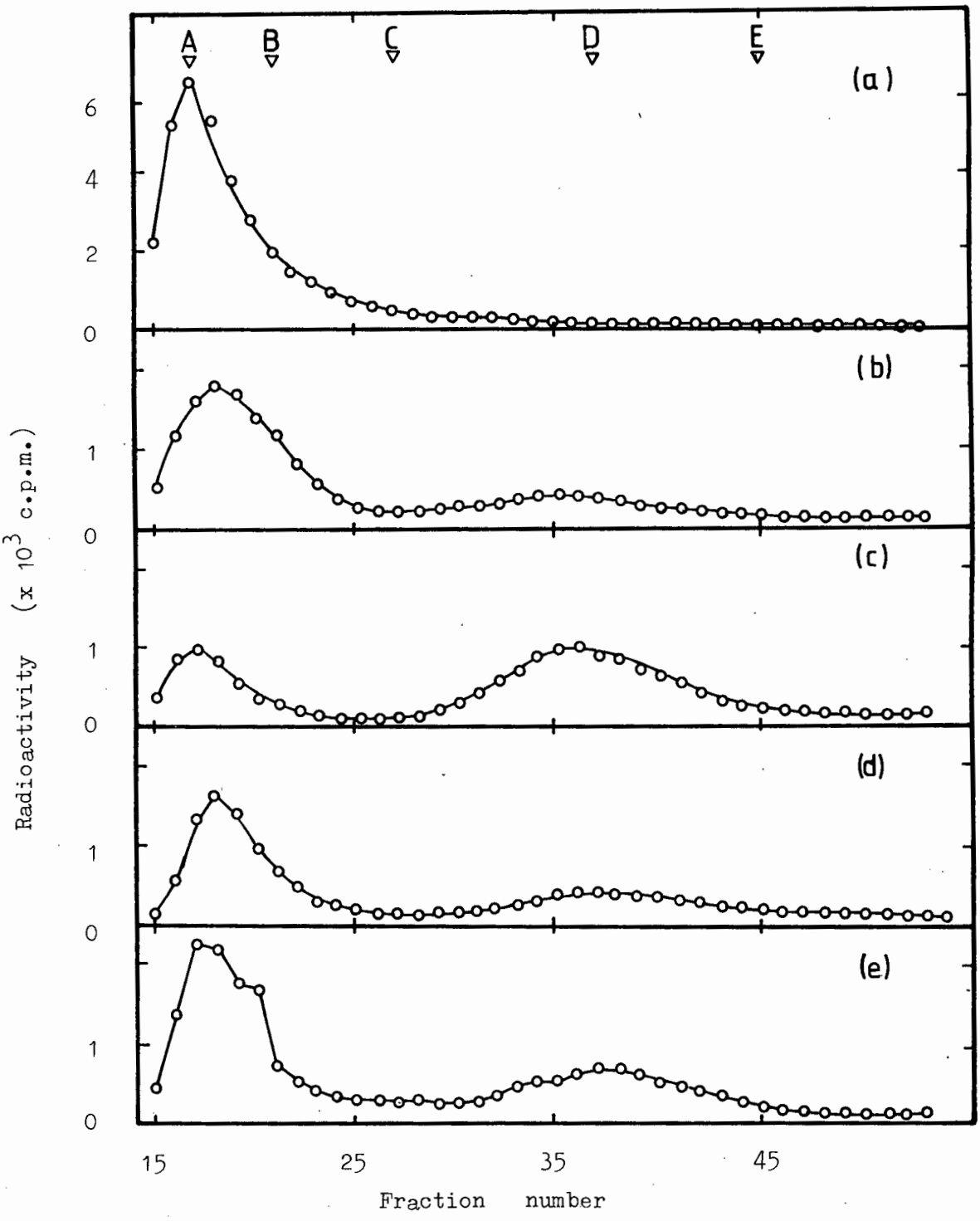


Fig. 3.4. Degradation of [^{14}C-me]globin by exponential phase Vibrio subcellular fractions.

The substrate, [^{14}C-me]globin was incubated with subcellular fractions for 60 min and 50 µl aliquots were passed through a Sephadex G-50 (0,6 x 5 cm) column. One drop fractions were collected and the radioactivity determined. Standards used were (A) blue dextran; (B) cytochrome C, M_r 12500; (C) insulin, M_r 6500; (D) Vitamin B12, M_r 2355; (E) phenol red, M_r 340. [^{14}C-me] globin without incubation with extract (a); Periplasmic fraction (b); Membrane fraction (c); ribosomal fraction (d); cytoplasmic fraction (e).

pH optimum between pH 7,0 and 9,5 but with marked inhibition below pH 7,0 (Fig. 3.3). Exponential extracts had optimum activity near pH 8,5.

3.3.3. Effect of protease inhibitors on proteolytic activities.

The effect of various inhibitors was determined in the absence of ATP (Table 3.2). All activities were markedly inhibited by the metal chelating agents EDTA and o-phenanthroline, and were more sensitive to o-phenanthroline, which preferentially chelates divalent cations other than Ca^{2+}. The serine protease inhibitor PMSF inhibited all the extracts greatly, approximately 80 % for both exponential and shaken stationary phase activities, and 65 % for the standing stationary phase activity. The thiol group inhibitor PHMB caused an inhibition of between 66 and 73 % for the stationary phase activities over a wide range of PHMB concentrations while the exponential phase activities were markedly less sensitive to PHMB, and the inhibition decreased with a decrease in PHMB concentration.

3.3.4. Subcellular localisation of proteolytic activities.

A modification of the method of Kaback (1971) was used to prepare periplasmic and cytoplasmic extracts (3.2.8). This method utilised lysozyme-EDTA to digest the cell wall, form spheroplasts and release periplasmic contents into the supernatant. Although the method has been found effective with a variety of bacteria (Kaback, 1971), its efficacy has been questioned with regard to stationary and exponential phase

cells growing in minimal media (Witholt et al., 1976). Apart from the presence of EDTA, Witholt et al. (1976) found that several conditions were needed before lysozyme could penetrate the outer membrane of stationary phase E. coli cells, namely the destabilisation of the outer membrane with a high concentration (200 mM) of Tris/HCl in the buffer, and mild osmotic shock to drive the lysozyme through the outer membrane. Spheroplast formation in Vibrio sp. 2 was essentially complete (> 90 % of the cells formed spheroplasts as judged by phase contrast microscopy) within 45 min of resuspension in TES buffer (3.2.8). This applied to exponential and both types of stationary phase cells. Neither mild osmotic shock nor a high concentration of Tris/HCl was found necessary, which might have been due to the resuspension of washed cells of halotolerant Vibrio sp. 2 (grown in 100 mM Tris/HCl with 400 mM NaCl) in TES buffer containing no salt but with 10 mM EDTA.

In all of the extracts prepared by the lysozyme-EDTA method, most of the activity was located in the cytoplasmic fractions (Table 3.3). Although the periplasmic preparations contained, in each case, a minor portion of the total activity, they showed a greater percentage stimulation by ATP. Preincubation did not affect the proteolytic activities of the various fractions in the presence or absence of ATP.

The method of Nossal and Heppel (1966) was used to repeat the experiments as this method required a much lower concentration of EDTA (10^{-4} M as opposed to 10 mM for the lysozyme-EDTA method), shown to inhibit Vibrio proteolytic activities (Table 3.2). Subcellular fractions were produced by differential centrifugation of cell-free extracts. The extracts were not examined to ascertain their purity,

although it was assumed that the P 30 fraction was membrane enriched, and that the S 100 fraction was essentially free of membrane material and polyribosomes.

The protein distribution in these subcellular fractions was determined (Table 3.4) and found to be similar in the various fractions for the three types of extract, except that the exponential phase periplasmic fraction had a significantly greater portion of the cells' protein than did the respective fractions of the stationary phase cells. Membrane fractions had a large portion of the cells' protein (exponential phase cells had 78 % and both types of stationary phase cells had 84 % of the cells' protein), but they did not have such a great portion of the cells' proteolytic activity against [^{14}C-me]globin (Table 3.5). Addition of 3 mM ATP significantly affected the distribution of proteolytic activity in standing stationary phase cell extracts. The periplasmic standing stationary phase extract had 53 % of the cells' proteolytic activity in the absence of ATP, and this decreased markedly in the presence of ATP, to 11 % of the cells' activity. Conversely, in the absence of ATP standing stationary phase cells had a small portion of the cells' activity in the soluble cytoplasmic fraction, and the proportion increased six-fold in the presence of ATP. The addition of ATP to extracts from shaken stationary phase cells did not change the distribution of activities significantly, and marginally changed that of the exponential phase cell extracts.

The large discrepancy between the periplasmic activity [between 9 and 25 % by the lysozyme-EDTA method (Table 3.3) and between 23 and 53 % by Nossal & Heppel's method (Table 3.5)] may be due to the different EDTA

concentrations used in the methods and the marked sensitivity of standing stationary phase extracts to EDTA (Table 3.2).

The activity of the various subcellular fractions against [^{125}I]-insulin was investigated in the absence of ATP (Table 3.6). ATP was not added as it had a negligible effect on the degradation of [^{125}I]-insulin by Vibrio extracts (Fig. 3.11), in contrast to the marked effect ATP addition had on globin degradation (3.3.5). In exponential phase, the major portion of insulin degrading activity was present in the periplasmic space (66 %) and the proportion was significantly smaller in stationary phase cell extracts. Conversely, the proportion of insulin degrading activity in both membrane and cytoplasmic fractions was higher in stationary phase cell extracts than in exponential phase cell extracts.

The insulin degrading activity in periplasmic fractions of exponential phase extracts showed minimal inhibition by 5 mM EDTA (Table 3.7), whereas the globin degrading activities in that fraction showed a 38 % decrease in activity with EDTA. Furthermore, while the cytoplasmic insulin-degrading activities were significantly inhibited by both EDTA and PMSF (and to similar extents), the globin degrading activities in that same fraction were not affected by either inhibitor.

These results are in contrast to those obtained with E. coli, as insulin degrading and globin degrading activities in E. coli have been shown to be separate, and activities against one of these substrates is either not active against the other or only very slightly so (Goldberg et al, 1981; Swamy & Goldberg, 1981; 1.5.4.). Furthermore, the globin (and

casein) degrading activities in E. coli are serine proteases (PMSF sensitive) while the insulin degrading activities are metallo-proteases and inhibited by metal ion chelators.

As these results showing globin degrading activities inhibited by EDTA in Vibrio sp. 2 were repeated in several independent experiments, it suggested that each fraction had a mixture of several activities which responded in a complex manner to the presence of inhibitors. The concentration of EDTA used was 5 mM, which has been shown to inhibit proteolytic activity against [^{14}C-me]globin by exponential phase extracts (Table 3.2), despite the presence of 10 mM Mg^{2+} in the buffers used to prepare the extracts, and indeed 1 mM EDTA inhibited stationary phase cell extract activities.

E. coli possesses a membrane associated proteolytic activity that degrades [^{35}S]-labelled denatured E. coli proteins to acid soluble peptides of mass 1500 or greater (Voellmy & Goldberg, 1981). The products of [^{14}C-me]globin degradation by the various Vibrio subcellular fractions was investigated by gel chromatography (Fig. 3.4). In each case, the substrate was degraded to material of mass between 700 and 3000, all of which was soluble in 5 % TCA. Similar experiments with [^{125}I]-insulin showed this substrate to have been degraded to material of mass 300 to 1000 (results not given). The presence of the labelled methyl groups on lysines in the substrate [^{14}C-me]globin may have prevented the complete degradation of this substrate to free amino acids, which may not have applied in the case of iodinated tyrosine in insulin.

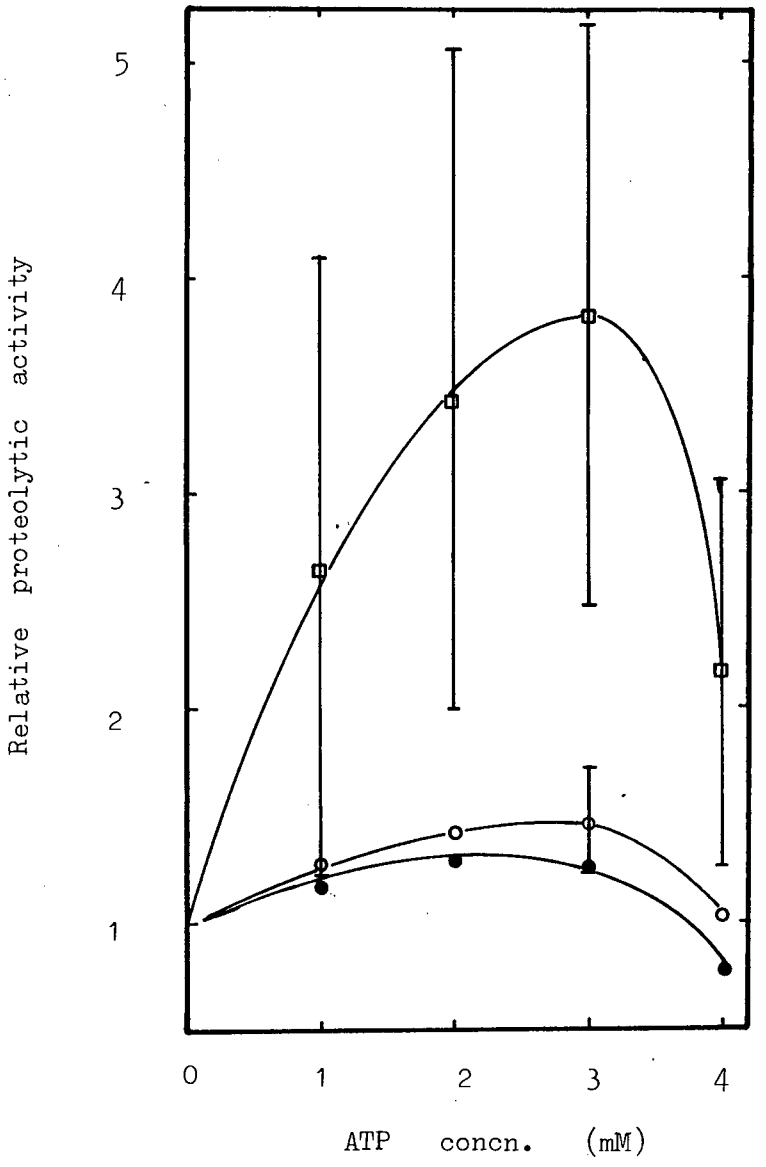


Fig. 3.5. Effect of ATP on the degradation of [^{14}C-me]globin by exponential and stationary phase Vibrio cell-free S30 extracts.

Proteolytic activity was determined in the presence of ATP as given (3.2.5) and expressed relative to that obtained without ATP addition. Preincubated dialysed cell extracts (3.2.2.1) from exponential phase (□); dialysed cell extracts from shaken (○) and standing (●) stationary phase cells. The points represent means from 20 experiments for the exponential phase extracts, and 5 experiments for the stationary phase extracts; the bars represent ranges of results. One bar for stationary phase extracts is given, the range of results for the other data being less than that given.

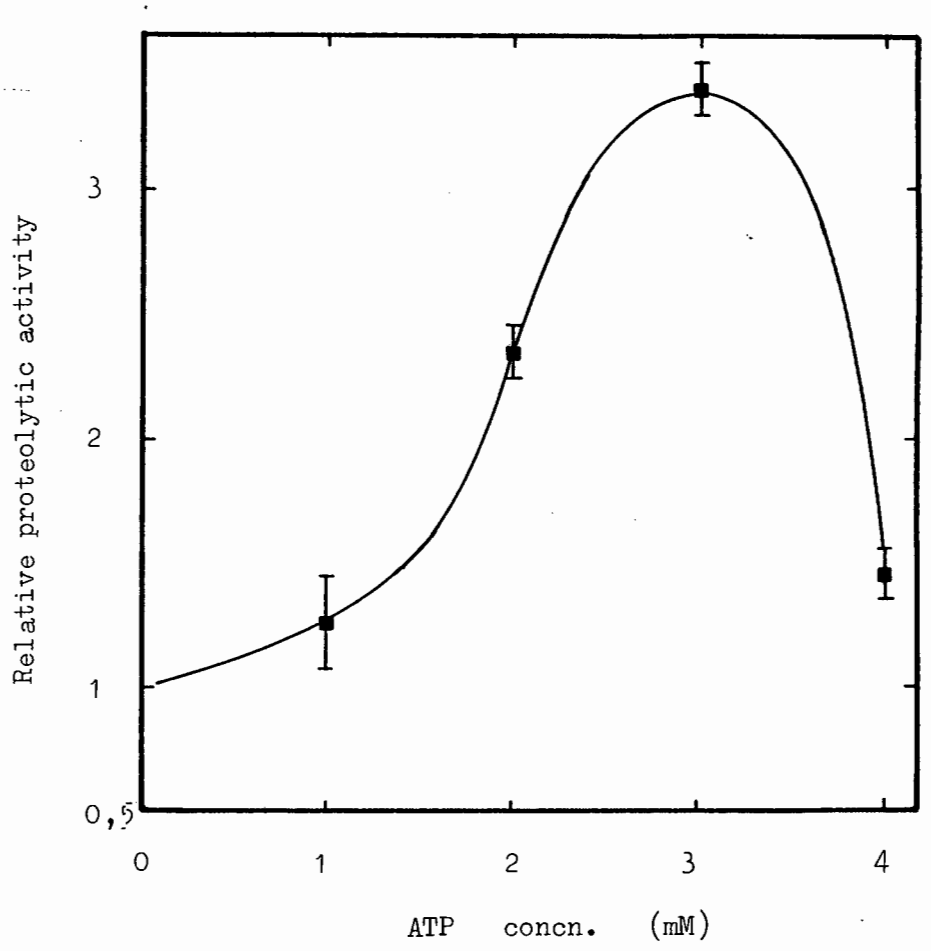


Fig. 3,6. Effect of preincubation on the degradation of [^{14}C-me]globin by exponential phase Vibrio cell-free (S 30) extract.

Aliquots of dialysed cell extract (3.2.2.1) were preincubated at 30 °C without ATP for 30 min before the addition of substrate with ATP. Activities are expressed relative to that obtained without ATP addition. Data were obtained from four separate experiments, but using different aliquots of the same cell-free preparation; the bars represent the range of results.

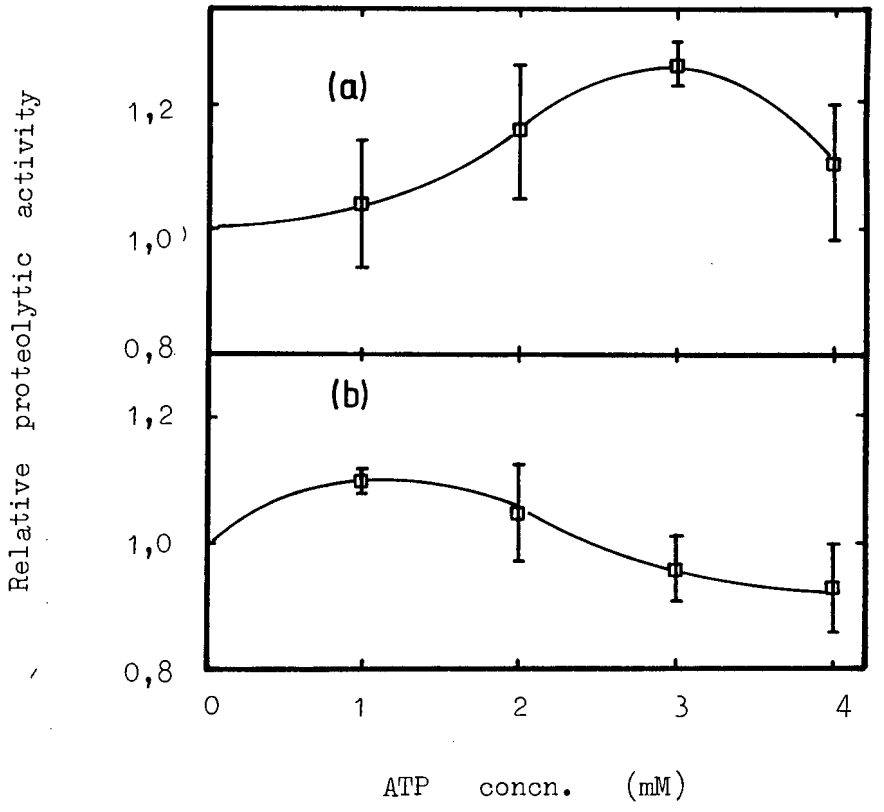


Fig. 3.7. Effect of ATP on the degradation of [^{14}C-me]globin by preincubated cell-free extracts from starving exponential cells.

Cells were starved (3.2.1) for (a) 30 and (b) 90 min by resuspension in growth limiting medium before preparation of the cell extracts. Proteolytic activity was determined in the presence of ATP as given (3.2.5) and expressed relative to that obtained without ATP addition. The points represent means from 3 experiments; the bars represent ranges of results.

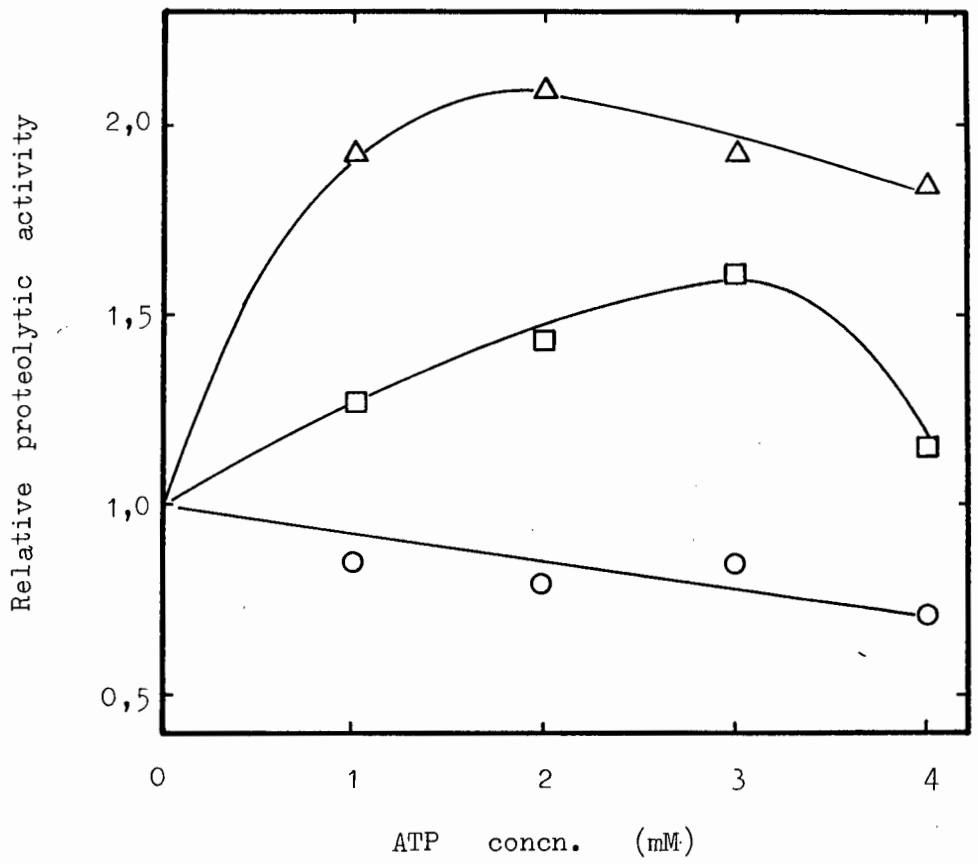


Fig. 3.8. Effect of ATP on the degradation of [14C-me]globin by exponential phase Vibrio cell-free extracts.

Proteolytic activity against [^{14}C-me]globin was determined in the presence of ATP as given (3.2.5) and expressed relative to that obtained without ATP addition. Cell lysis was by (△) YEDA press; (□) lysozyme-EDTA and (○) grinding with alumina. The results are from one experiment, repeat experiments gave similar results. The extracts were not preincubated.

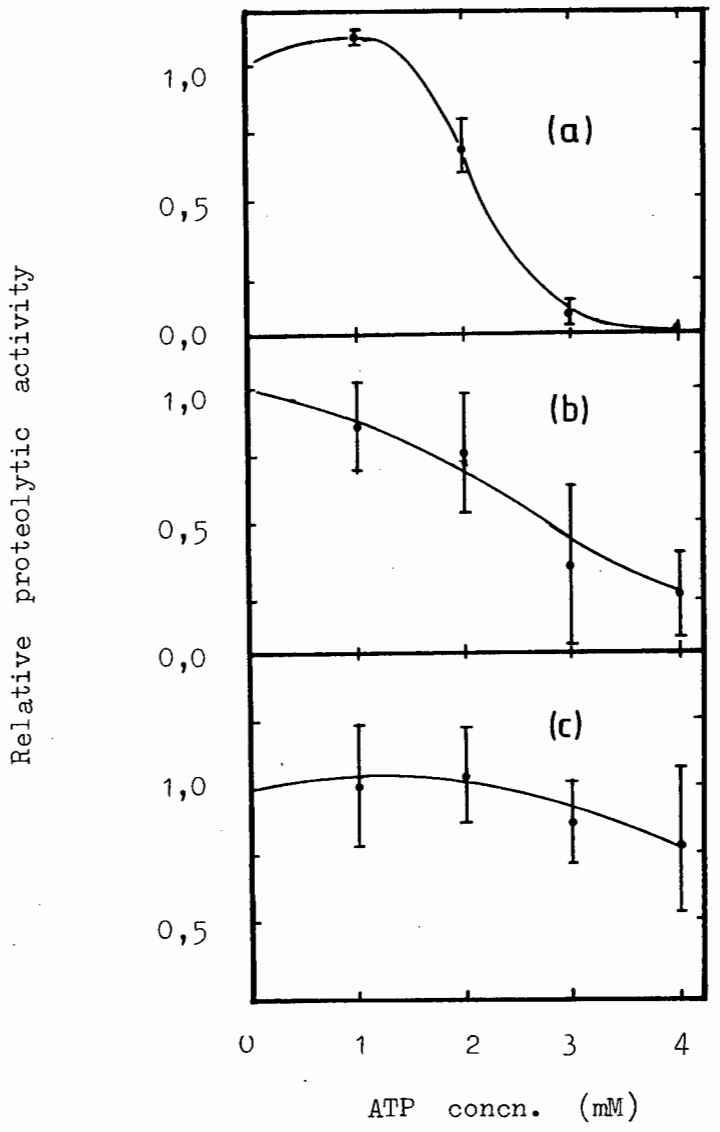


Fig. 3.9. Effect of ATP on the degradation of [^{14}C-me]globin by exponential phase Vibrio cell-free extracts prepared by grinding with alumina.

Proteolytic activity against [^{14}C-me]globin was determined in the presence of ATP as given (3.2.5) and expressed relative to that obtained without ATP addition, for the subcellular (3.2.8) preparations (a) P 30; (b) P 100; and (c) S 100. The points are means of three independent experiments, the bars represent the ranges of results.

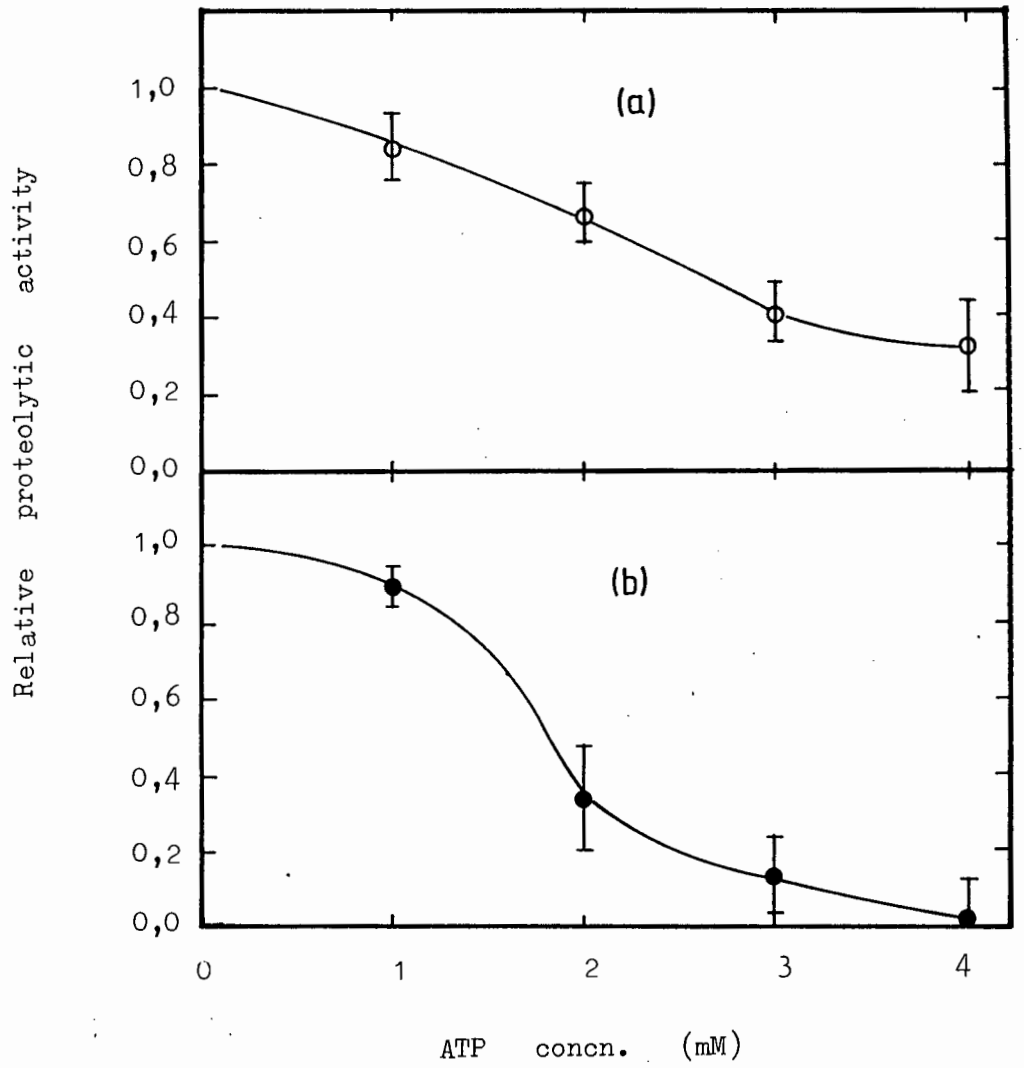


Fig. 3.10. **Effect of ATP on the degradation of [14C-me]globin by stationary phase cell extracts produced by grinding with alumina.**

Proteolytic activity against [^{14}C-me]globin was determined in the presence of ATP as given (3.2.5) and expressed relative to that obtained without ATP addition, for (a) shaken stationary and (b) standing stationary phase cell-free P 30 extracts. The points are the means of four independent experiments; the bars represent the ranges of results.

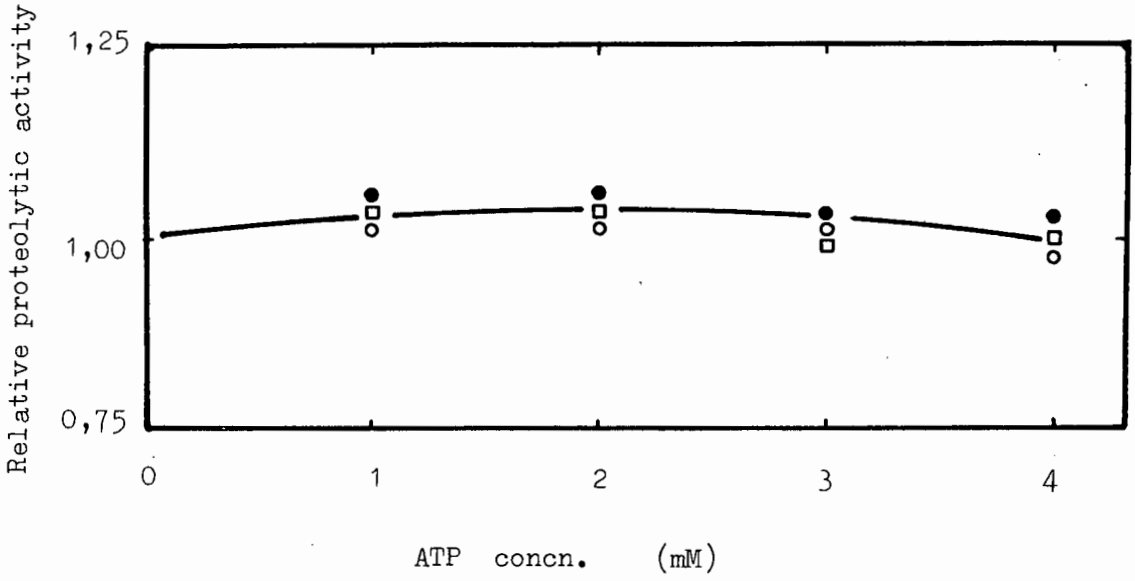


Fig. 3.11. Effect of ATP on the degradation of [^{125}I]-insulin by Vibrio cell-free extracts.

Proteolytic activity against [^{125}I]-insulin in S 30 extracts (3.2.2.1) was determined in the presence of ATP as given (3.2.5) and expressed relative to that obtained without ATP addition, for (□) exponential; (○) shaken stationary and (●) standing stationary phase extracts. These results are from one of three independent experiments, all showing similar results.

It was not possible to assess the amount of non-radioactive protein present in the various subcellular fractions that served as competing substrates, hence the per cent values measured may not have corresponded exactly with the situation *in vivo*.

3.3.5. Effect of ATP on proteolytic activities.

The addition of ATP to *Vibrio* cell-free extracts affected [^{14}C-me]globin degradation (relative to globin degradation without added ATP) in several ways, depending on the procedure used to produce cell lysis, the conditions of assay (whether the extract was preincubated or not), and the cultural conditions of the cells.

Extracts (S 30) from exponential and both types of stationary phase cells produced by the lysozyme-EDTA method were stimulated by ATP (Table 3.8) to approximately the same extent by 3 mM ATP. Preincubation of the exponential phase extract increased the stimulation of proteolytic activity markedly, from < 2-fold for non-preincubated extracts (Table 3.8) to between 2,5- and 5,1-fold (Fig. 3.5). Stationary phase extracts were not affected in this way by preincubation (Table 3.8), and the stimulation was reduced by approximately 20 %.

The amount of ATP-stimulation observed in preincubated exponential phase extracts varied greatly (Fig. 3.5) between repeat experiments using independently produced extracts, whereas the variation was not as large in repeat experiments using different aliquots of the same cell-free preparation (Fig. 3.6). Extracts (S 30) produced from starving

exponential phase cells (Fig. 3.7) were stimulated < 1,3-fold by the addition of ATP, and this stimulation was not affected by preincubation.

The method of cell lysis influenced the effect ATP addition had on [^{14}C-me]globin degradation. Both the lysozyme-EDTA and Yeda press methods yielded Vibrio extracts that were stimulated by ATP addition, however extracts prepared from cells lysed by grinding with alumina produced S 30 preparations depressed by ATP (Fig. 3.8). ATP itself binds magnesium strongly, and may have affected proteolytic activity,as with chelating agents (3.3.3) although ATP was present in the assay system at 3mM final concentration, and $MgCl_2$ at 10mM.

Crude cell extracts prepared from cells lysed by grinding with alumina were subjected to differential centrifugation to produce membrane enriched (P 30), intermediate ('ribosomal', P 100), and membrane-free (soluble cytoplasmic, S 100) fractions (3.2.8). The proteolytic activities in all these fractions were depressed by the addition of ATP (Fig. 3.9), with the membrane enriched fraction the most depressed, and the soluble cytoplasmic fraction the least depressed.

The proteolytic activities in extracts produced from both types of stationary phase cells by grinding with alumina were also depressed by the addition of ATP (Fig. 3.10).

In contrast to the effects ATP addition had on Vibrio sp. 2 cell-free extracts, the degradation of [^{125}I]-insulin in any of these extracts was not affected by the addition of ATP. The results for insulin degradation by S 30 extract in the presence of ATP is given (Fig. 3.11).

Nucleotides other than ATP (dATP, GTP, ADP and AMP) were tested, but only GTP showed any effect on the substrates used. In exponential cell

extracts, GTP (1 and 2 mM) caused a 25 % and 13 % decrease respectively in the proteolytic activities, and the stationary phase extracts showed less than 7 % increase in proteolytic activity with 1 and 2 mM GTP. However, as the level of GTP in both types of stationary phase is low (2.3.3) the physiological significance of this stimulation by GTP is not clear.

3.4 Discussion.

The specific proteolytic activities of the three types of cell-free extract varied less than 20 % between the extracts, with the activity of shaking stationary phase cell-free extracts against [^{14}C-me]globin higher, and against [^{125}I]-insulin lower than that of the other two types of extract. However, in vivo protein turnover varied from about 1 to 20 % h^{-1} among the three different phases (2.3.2). Murakami et al., (1979) found 2- to 3-fold differences in proteolytic activities between the extracts of E. coli deg^+ and deg^- strains. By a variety of other criteria however, the proteolytic activities responsible for the degradation of these substrates in Vibrio sp. 2 cell-free extracts appear to be different in each of the three types of extract. The addition of various protease inhibitors, ATP addition and the subcellular distribution of proteolytic activities showed that there were differences between the three types of extract.

The method of extract preparation, (Fig. 3.8) extract treatment (that is, whether the extracts were preincubated or not, (Table 3.8) and the culture conditions prior to extraction (Figs. 3.5 & 3.7) all affected [^{14}C-me]globin degradation in the presence of ATP. The degradation of the substrate [^{125}I]-insulin was not significantly affected by the addition of ATP. Goldberg et al., (1981) found that in E. coli

extracts, large molecule substrates, such as apohaemoglobin and casein were degraded by an ATP-dependent system, while small proteins, such as insulin, were not.

Vibrio sp. 2 cell-free extracts that were essentially free of cell wall material (S30) from exponential phase cells showed a large (2- to 5-fold) stimulation by 3 mM ATP after the extracts had been preincubated in the absence of ATP. Without preincubation, these extracts showed a mild (< 2-fold) stimulation of proteolytic activity. As the results were reproducible in repeat experiments using aliquots of the same extract, but varied considerably between independently produced extracts, it suggested that there may be several factors involved in producing this effect, and that various treatments affected the factors in different ways. Cell lysis achieved with the use of alumina (3.2.2.3) abolished the stimulatory effect of ATP on proteolysis, and instead caused a marked repression by ATP (Figs. 3.9 & 3.10), particularly in fractions enriched with cell membrane (P30).

The addition of ATP has been found to stimulate the degradation of abnormal or foreign proteins in reticulocyte lysates (1.5.2 & 1.5.5) and in E. coli extracts (Murakami et al., 1979), however repression of proteolytic activities by ATP does not appear to have been reported previously. The amount of variation obtained in the experiments with E. coli extracts was not reported, although Liu et al. (1984) reported similar variations to those found in this study in their studies on abnormal protein degradation in extracts of yeast.

The shaken and standing stationary phase proteolytic systems responsible for the degradation of [^{14}C-me]globin showed a response similar to that of exponential phase extracts in that cell wall- and membrane-rich fractions (P30) for which the cells were lysed by grinding with alumina were also depressed by the addition of ATP (Fig. 3.10) while cytoplasmic fractions were stimulated < 2-fold (Fig. 3.5 & Table 3.8). However, preincubation did not increase the degree of ATP stimulation and in this respect these extracts resembled those prepared from starving exponential phase cells (Fig. 3.7).

The stimulation of [^{14}C-me]globin degradation in S30 preparations appeared maximal in the presence of 3 mM ATP [Figs. 3.5; 3.6 & 3.7 (a)] which is approximately the physiological concentration of this nucleotide (Olden & Goldberg, 1978), although *Vibrio* extracts had to be preincubated before ATP stimulation was observed. In *E. coli* the stimulatory effect of ATP was also maximal at 3 mM ATP, but the effect was lost after preincubation (Murakami *et al.*, 1979). On prolonged starvation of exponential *Vibrio* cells prior to extraction [Fig. 3.7 (b)] the maximum stimulation by ATP was between 1 and 2 mM. Depression of proteolytic activity by ATP also appeared to be maximal for 3 to 4 mM ATP (Figs. 3.9 & 3.10). This is interesting as the level of ATP in stationary phase *Vibrio* cells has been shown to be between 20 and 57 % lower than in exponential phase cells (Robb, 1979; Robb *et al.*, 1980). St. John & Goldberg (1978) reported that a moderate inhibition of energy metabolism (30 to 50 % reduction) accelerated the breakdown of normal cell proteins in a similar fashion to starvation, but a drastic reduction of ATP levels (80 % reduction) inhibited the degradation of all cell proteins.

A factor that could not be controlled for in these experiments was the amount of substrate present within the extracts that competed with the radiolabelled substrates used, although at the concentrations of protein in the extracts used in these studies (10 mg ml^{-1} protein) the degradation of radiolabelled substrates was essentially linear (Figs. 3.1 & 3.2) over the times these assays were performed.

CHAPTER FOUR.

INTRACELLULAR PROTEOLYTIC ACTIVITIES.

4.1. Introduction.

The ATP-dependent protease in E. coli (protease La; 1.5.4) is the 94 $x10^3$ dalton polypeptide product of the lon gene (capR) which has been highly purified (Charette et al., 1981). The proteolytic activity is ATP dependent, and hydrolyses [^{3}H]-labelled casein into TCA soluble fragments. Although ATP was the preferred nucleotide, CTP and UTP could substitute for ATP, but with less efficiency. In addition to this protease, E. coli has several other serine proteases, and at least two metallo-proteases, none of which are affected by added ATP (Swamy & Goldberg, 1981; 1.5.4).

In contrast to the E. coli ATP-dependent activity, the soluble ATP-dependent (non-lysosomal) proteolytic activity in rabbit reticulocytes is a multi-component system, in which the protease, ATP, ubiquitin, and an inhibitor (Speiser & Etlinger, 1983) have a role (1.5.5).

Proteolysis in extracts of Vibrio sp. 2 was either stimulated or repressed by the addition of ATP, depending on the method of cell lysis and the centrifugation protocol used to prepare the extract (Ch. 3). This suggested that Vibrio cells may have contained separate ATP-stimulated and ATP-repressed activities, or an activity that responded

to the presence of ATP in a complex manner depending on other factors (such as inhibitors) within the system.

Experiments were undertaken to investigate the nature of the ATP-affected proteolytic activity in Vibrio sp..2 extracts.

4.2. Materials and methods.

The strain, growth conditions, proteolytic assay and extraction procedures were as described (2.2 and 3.2) with minor variations detailed below.

4.2.1. Protease assay.

[^{14}C-me]Globin and [^{125}I]-insulin were used as described (3.2.4 and 3.2.5). Column fractions were assayed by incubating 450 µl of the fraction with 50 µl of substrate (approximately 10^4 c.p.m.) in the presence or absence of 3 mM ATP at 30 °C for 60 min. The reaction was stopped by adding 25 µl of TCA (100 % w/v), mixed rapidly, and 25 µl of carrier bovine serum albumin (BSA; 30 mg ml^{-1}) was added. After 30 min the samples were centrifuged and acid soluble radioactivity determined. Blanks were also assayed, using 450 µl of TM saline in place of the sample, to give the acid soluble radioactivity initially present, which was subtracted from the data obtained.

4.2.2. Preparation of cell-free extracts.

Extracts were prepared as described (3.2.2), after the cells had been lysed using the YEDA press (3.2.2.2). Subcellular fractions were obtained essentially as described (3.2.8). Unbroken cells and debris were removed by centrifugation (3 000 g; 20 min) and the resulting supernatant, S 3 was considered to be the whole cell extract, from which P 30 (the pellet after centrifugation at 30 000 g for 30 min) and P 100 (the pellet after centrifugation at 100 000 g for 2 h) were prepared, and were considered to be membrane enriched. A soluble cytoplasmic fraction, S 100, was the supernatant produced with the P 100, and was considered to be essentially membrane-free. The pellets were resuspended by adding buffer (TMM saline) to the original volume, and homogenising in a Braun homogeniser.

4.2.3. Protein concentrations.

Protein concentrations were determined as given (3.2.3) or by measuring the absorbance at 280 nm. The absorbance was measured in each fraction or by using a recording spectrophotomer as indicated in the figure legends.

4.2.4. Exclusion chromatography.

Exclusion chromatography beds were prepared according to the manufacturers' recommendations. Sephacryl S-100, S-200 and S-1000 superfine were packed in 0,9 x 60 cm columns and equilibrated with TMM

saline. Sephadex gels were prepared from the powders as recommended, degassed, packed in columns, and operated at the recommended flow rates.

4.2.5. Ion exchange chromatography.

Whatman DE52 preswollen microgranular DEAE cellulose was prepared according to the manufacturer's recommendations (Whatman information leaflet), equilibrated thoroughly, degassed, packed and washed with TMM extensively before use. Samples were dialysed, the salt concentration determined and adjusted to < 5 mM salt if necessary by dilution. After sample loading (by gravity) the column was washed extensively and eluted with salt gradients as given. Salt concentration was determined by the Mohr titration (Vogel, 1961).

4.2.6. Preparation of inner and outer membrane fractions.

Isopycnic centrifugation was used, following the method of Osborn & Munson (1974) to separate the inner and outer membranes, and to prepare a 'membrane' fraction. The membrane fraction (P 30; 4.2.2) was homogenised in a Braun homogeniser, and protease free DNase I (Boehringer Mannheim, c. 40 μg ml^{-1}) was added to reduce the viscosity due to DNA. The preparation was incubated on ice for 40 min (the viscosity was visibly reduced) and diluted. The membrane fraction was again centrifuged (100 000 g; 60 min), resuspended in TEAS using a Braun homogeniser, and layered (5 mg protein per tube) onto a 40 - 70 % sucrose (in Tris/HCl 30 mM; pH 8,0 with 50 mM EDTA) block gradient and centrifuged in a Beckmann SW 27,0 rotor at 20 000 r.p.m. for 16 h at 4

^{o}C. Bands were harvested by suction using a blunt needle, diluted in TMK buffer and precipitated by centrifugation at 100 000 g for 60 min. The pellets were resuspended in the original volumes of TM buffer using a Braun homogeniser, and stored at -70 ^{o}C in 1 ml aliquots.

4.2.7. Precipitation with ammonium sulphate.

Precipitation with ammonium sulphate was used to concentrate proteins. Solid ammonium sulphate, finely ground in a mortar and pestle, was slowly added to the protein solution with stirring at 4 ^{o}C, allowing each addition to dissolve before adding more solid. The solution was stirred for 1 h before centrifugation (15 000 g; 20 min). The pellets were dissolved in a minimum of TM buffer and dialysed extensively with several changes of buffer. The quantities of ammonium sulphate required were determined using a nomograph (Dixon, 1953).

4.2.8. Ultrafiltration.

Pooled fractions were concentrated by ultrafiltration using Millipore PTGC filters (nominal limit 10 x10^3) at the recommended pressure. The whole procedure was performed with the device packed in ice.

Table 4.1. Ammonium sulphate precipitation of exponential phase Vibrio S 30 preparation.

A sample (10 ml) of an S 30 preparation (10 mg protein) originally showing 1,5 to 2 fold stimulation by ATP was fractionated by ammonium sulphate precipitation. The cuts were dissolved in minimum TM buffer, dialysed extensively against TM saline, then adjusted to the initial volume with TM saline. Proteolytic activity against [^{14}C-me]globin, with and without 3 mM ATP was measured, as well as the protein concentration (expressed as a percentage of the total amount of protein), in each cut.

Ammonium sulphate (w/v)	Proteolytic activity without ATP (% of total activity)	Protein (% of total)	Stimulation by ATP (3mM)
20	22	4	1,04
40	14	4	0,93
60	33	40	0,75
80	30	43	0,86
100	1	8	1,00

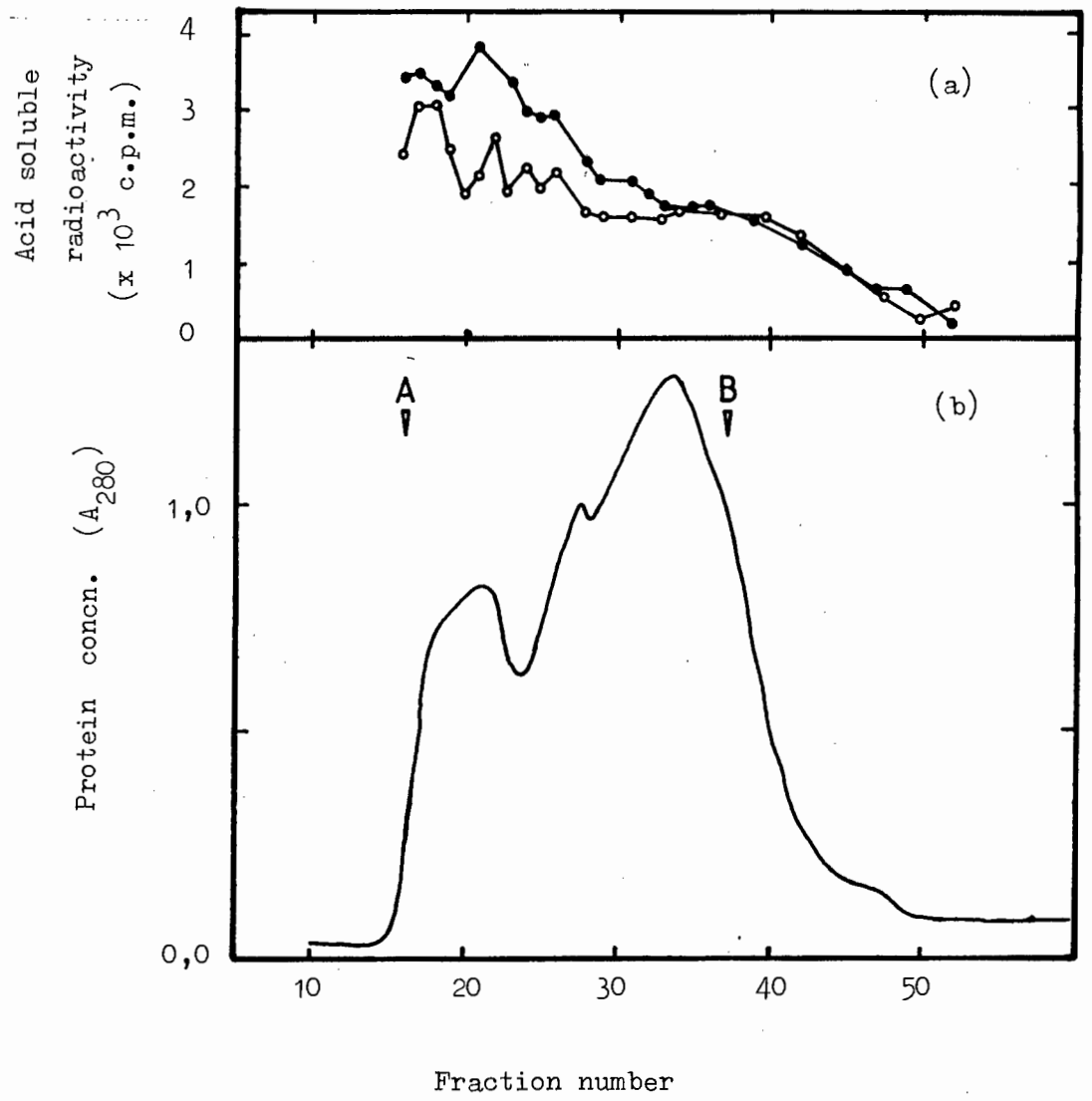


Fig. 4.1. Exclusion chromatography of exponential phase Vibrio whole cell extract (S 3).

An aliquot (1 ml) of Vibrio S 3 extract, from a lysate produced by grinding with alumina, was loaded onto a Sephacryl S-1000 column (0,9 x 60 cm) and eluted with TM saline. Fractions (0,85 ml) were assayed for (a) [^{14}C-me]globin degradation with (o) and without (•) 3 mM ATP. Protein elution (b; A_{280}) was followed using a recording spectrophotometer. Markers used were (A) whole cells and (B) haemoglobin. Similar results were obtained with stationary phase Vibrio extracts, and when the gel used was Sepharose 4B.

4.3. Results.

4.3.1. Whole cell extracts.

Whole cell extracts were fractionated by several techniques in order to separate ATP affected activities. Initial attempts to detect Vibrio intracellular proteases using gelatin- or haemoglobin- SDS polyacrylamide gel electrophoresis (Heussen & Dowdle, 1980) were unsuccessful, possibly due to the low proteolytic activity present in intracellular preparations, as undiluted aliquots spotted directly onto the substrates (copolymerised in the gels) did not cause detectible degradation of the substrate.

Ammonium sulphate precipitation of a Vibrio S 30 preparation caused the loss of ATP stimulation (Table 4.1), which limited the use of this technique. ATP may have affected proteolysis indirectly, as has been observed in reticulocytes (1.5.6) where ubiquitin, and possibly another factor, an inhibitor (Speiser & Etlinger, 1983) are involved with ATP in modulating endoproteolysis. Proteolysis by various mixtures of the ammonium sulphate fractions was measured both with and without ATP, but no mixture was found to be affected by ATP.

Whole cell extract (S 3) produced from cells lysed by grinding with alumina was chromatographed on Sephacryl S-1000 (exclusion limit several hundred million; Fig. 4.1). The ATP depressed activity eluted with the large particulate fraction (larger than haemoglobin), as was most of the 280 nm absorbing material (Fig. 4.1). Membrane enriched fractions (P30)

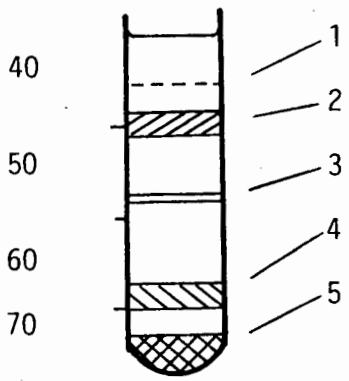
Table 4.2. Proteolytic activity of the inner and outer membranes of exponential phase *Vibrio* sp. 2 cells.

Inner and outer membranes were separated according to the method of Osborn & Munson (1974). The proteolytic activity against [^{14}C-me]globin of the P 30 preparation was originally stimulated 1,5 to 2 fold by ATP. The P 30 preparation was used as described (4.2.6) and 3 bands, upper (U; 'inner' membrane), lower (L; 'outer' membrane) and middle (M; a mixture of both) were harvested, washed by centrifugation and resuspended in the original volume of TM buffer. Proteolytic activities are expressed relative to the U band, and the activity of the P 30 preparation was 4,5 times that of the U band (determined in the absence of ATP).

Sample	Protein concn. (mg ml^{-1})	Relative proteolytic activity (- ATP)	Relative proteolytic activity (+ATP/-ATP)
U	1,10	1,00	1,06
M	0,67	1,80	0,77
L	8,50	1,80	0,93

Table 4.3. Proteolytic activities of fractionated cell envelope.

Exponential phase Vibrio membrane-enriched preparation (P 30) was fractionated according to the method of Osborne & Munson (1974), except that no EDTA was used in the buffers. The preparation was centrifuged on a sucrose block gradient, and the proteolytic activity against [^{14}C-me]globin was determined, with and without 3 mM ATP. Proteolytic activities are expressed as a percentage of the total proteolytic activity. ND - not determined. The effect of ATP is expressed as the ratio of activity in the presence of ATP to the activity in the absence of ATP.



Bands	Density ($g\ ml^{-1}$)	Relative proteolytic activity (%)	Effect of ATP
1	1,12	2	1,48
2	1,16	16	1,19
3	1,20	20	1,27
4	1,23	35	0,56
5	ND	27	0,79

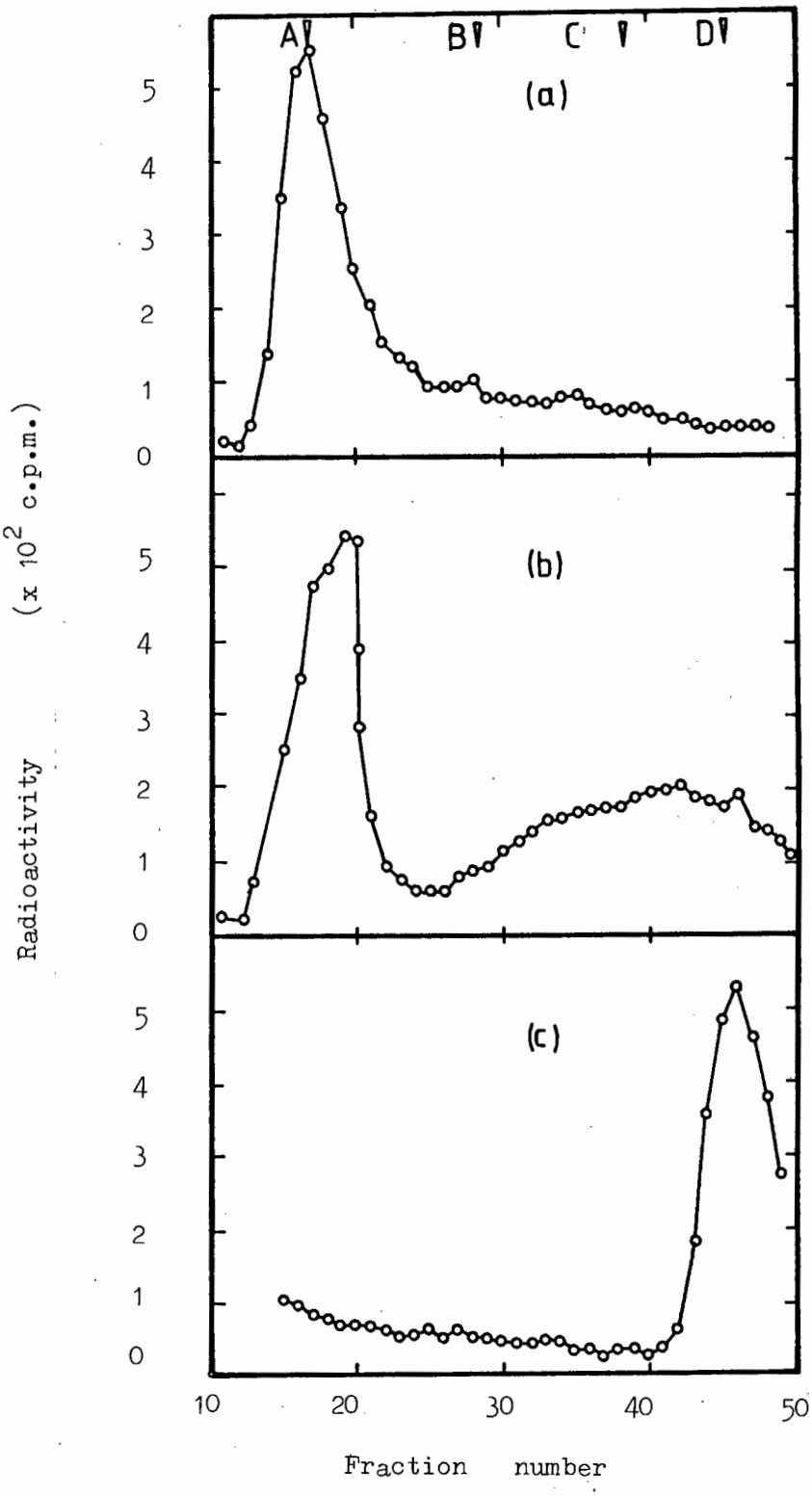


Fig. 4.2. Exclusion chromatography of [^{14}C-me]globin degradation products formed by exponential phase Vibrio cell envelope preparation.

Cell envelope (band 3; Table 4.3), isolated by the method of Osborne & Munson (1974) without using EDTA was incubated with [^{14}C-me]globin without the addition of ATP for (a) 0 min; (b) 60 min and (c) 16 h before an aliquot (50 µl) of the reaction mixture was run on a Sephadex G-50 column (0,6 x 5 cm). The radioactivity in one drop fractions was determined. Markers used were (A) blue dextran; (B) insulin, M_r 6500; (C) vitamin B12, M_r 1355 and (D) phenol red, M_r 350.

Table 4.4. Separation of intrinsic and extrinsic membrane proteolytic activities from Vibrio.

Several 'chaotropic agents' were used (Bennett, 1982) to solubilise peripheral membrane proteolytic activities. Solid agent was added to an aliquot (0,5 ml) of band 3 (Table 4.3), the volume was made up to 2,0 ml with water and the solid dissolved. The control had no solid material added. The membrane fractions were precipitated by centrifugation and diluted to 2,0 ml in TM buffer after resuspension using a Braun homogeniser. All samples were dialysed against TM saline and the activity against (A) [^{14}C-me]globin and (B) [^{125}I]-insulin determined. Addition of 3 mM ATP gave results similar to those given, within 15 %.

A Proteolytic activity against [^{14}C-me]globin

Chaotropic agent	Supernatant: acid soluble c.p.m.	Supernatant: portion of activity	Membrane: acid soluble c.p.m.	Membrane: portion of activity	Total activity (spn. + memb.) relative to control.
nil(cont.)	1720	0,62	1070	0,38	1,00
NaCl (1 M)	288	0,23	961	0,77	0,45
NaI (2 M)	1489	0,65	814	0,35	0,83
Urea (8 M)	766	0,56	600	0,44	0,49

B Proteolytic activity against [^{125}I]-insulin

Chaotropic agent	Supernatant: acid soluble c.p.m.	Supernatant: portion of activity	Membrane: acid soluble c.p.m.	Membrane: portion of activity	Total activity (spn. + memb.) relative to control
nil(cont.)	1064	0,53	943	0,47	1,00
NaCl (1 M)	1059	0,54	920	0,46	0,99
NaI (2 M)	749	0,45	907	0,55	0,83
Urea (8 M)	2243	0,71	916	0,29	1,57

prepared using S3 preparations, were markedly repressed by the addition of ATP [Fig. 3.9 (a)].

4.3.2. Membrane proteolytic activity.

E. coli cell envelope material may be isolated from cellular material by isopycnic centrifugation (Osborn & Munson, 1974) using a sucrose gradient. The presence of 5 mM EDTA in the sucrose gradient furthermore allows the separation of the cell envelope into 3 bands, an upper band (the cytoplasmic membrane, at density 1,16 g ml^{-1}), a lower band (outer membrane, at density 1,22 g ml^{-1}) and a band at intermediate density (1,19 g ml^{-1}) consisting of unseparated envelope fragments.

Vibrio whole envelope preparations (P 30) fractionated using this method, gave 3 discrete bands at the appropriate buoyant densities (1,16; 1,20 and 1,22 g ml^{-1}) which were harvested by aspiration using a blunt needle (Table 4.2). The proteolytic activity of each band was low compared to the activity of the original P 30 preparation, and the bands U and L showed no significant effect with the addition of 3 mM ATP. In the presence of ATP, the intermediate band, M, showed a reduction of proteolytic activity to 0,77 of that measured in the absence of ATP.

Factors affecting the separation of inner and outer membranes using isopycnic centrifugation include the presence of divalent cations, hence the use of 5 mM EDTA in the procedure (Osborn & Munson, 1974). However, as EDTA significantly affected Vibrio intracellular proteolytic activities (3.3.3) the procedure was repeated [after the P 30 preparation had been washed by centrifugation (100 000 g; 60 min) in 10

mM Tris/HCl (ph 8,0)] without EDTA. Four bands and a precipitate were observed (Table 4.3). The first, very faint band (band 1) formed at a buoyant density of 1,12 g ml^{-1}. Protein degradation in this band was stimulated 1,48-fold with 3 mM ATP, however as it was at a low buoyant density, may not be part of the membrane associated activity. Band 2 was a broad, diffuse band, which had a significant portion of the total proteolytic activity, and showed slight stimulation with ATP. It banded at a buoyant density of 1,16 g ml^{-1}, at which density E. coli cytoplasmic membrane material forms a band (1,14 to 1,16 g ml^{-1}; Osborn & Munson, 1974). Band 3 was a thin dense band, at a density of 1,20 g ml^{-1} and had a significant portion of the membrane's proteolytic activity (20 %) which was stimulated 1,27 fold with 3 mM ATP. Band 3 probably represented a mixture of inner and outer membrane material, as it banded at an intermediate density between those observed for inner (1,16 g ml^{-1}) and outer (1,22 g ml^{-1}) membrane material from E. coli. Band 4 was a broad, dense band, at a buoyant density of 1,23 g ml^{-1}, and had a significant portion (35 %) of the total membrane proteolytic activity, which was depressed 0,56 fold with addition of ATP. This band probably represented the outer membrane fraction of the preparation, however it was adjacent to the precipitate, band 5, which sedimented into the 70 % sucrose 'cushion', and may have been contaminated by material from that band during harvesting. The precipitate, band 5, had a significant portion of the total proteolytic activity, which was also depressed by ATP. This last band possibly contained cell debris and wall material.

The purity of the putative inner and outer membrane preparations was not established, as convenient marker enzymes were not available for Vibrio sp.2.

The degradation of [^{14}C-me]globin by the band 3, Table 4.3 (considered to be a mixture of inner and outer membranes), was analysed by exclusion chromatography on Sephadex G-50 (Fig. 4.2). The substrate was considerably degraded to fragments with heterogeneous $\underline{M}_r$ in the range 5 000 to 300 within 60 min [Fig. 4.2 (b)], and after 16 h the radioactivity was found in material of $\underline{M}_r$ less than 300 [Fig. 4.2 (c)].

Membrane-associated enzymes are either intrinsic or peripherally attached to the membrane. Peripheral membrane proteins may be separated from the membrane using 'chaotropic' agents (Bennett, 1982). Treatment of band 3 (Table 4.3) with chaotropic agents caused the loss of the effect ATP had on proteolysis. Washing band 3 with deionised water redistributed the [^{14}C-me]globin degrading activity, approximately 60 % in the supernatant, approximately 40 % remaining with the membrane fraction, and distributed the [^{125}I]-insulin degrading activity approximately equally between the fractions (Table 4.4). NaCl (1 M) markedly reduced the amount of [^{14}C-me]globin degrading activity that became solubilised, and also reduced the total activity in both the supernatant and membrane fractions. Urea (8 M) also reduced the total amount of globin degrading activity, by a half, but did not cause the redistribution of activity to be markedly different from that obtained with water alone. Urea enhanced the total [^{125}I]-insulin degrading activity in band 3 [Table 4.4 (B)] relative to treatment with water alone, by doubling the activity found in the supernatant, while not markedly affecting that found in the membrane fraction.

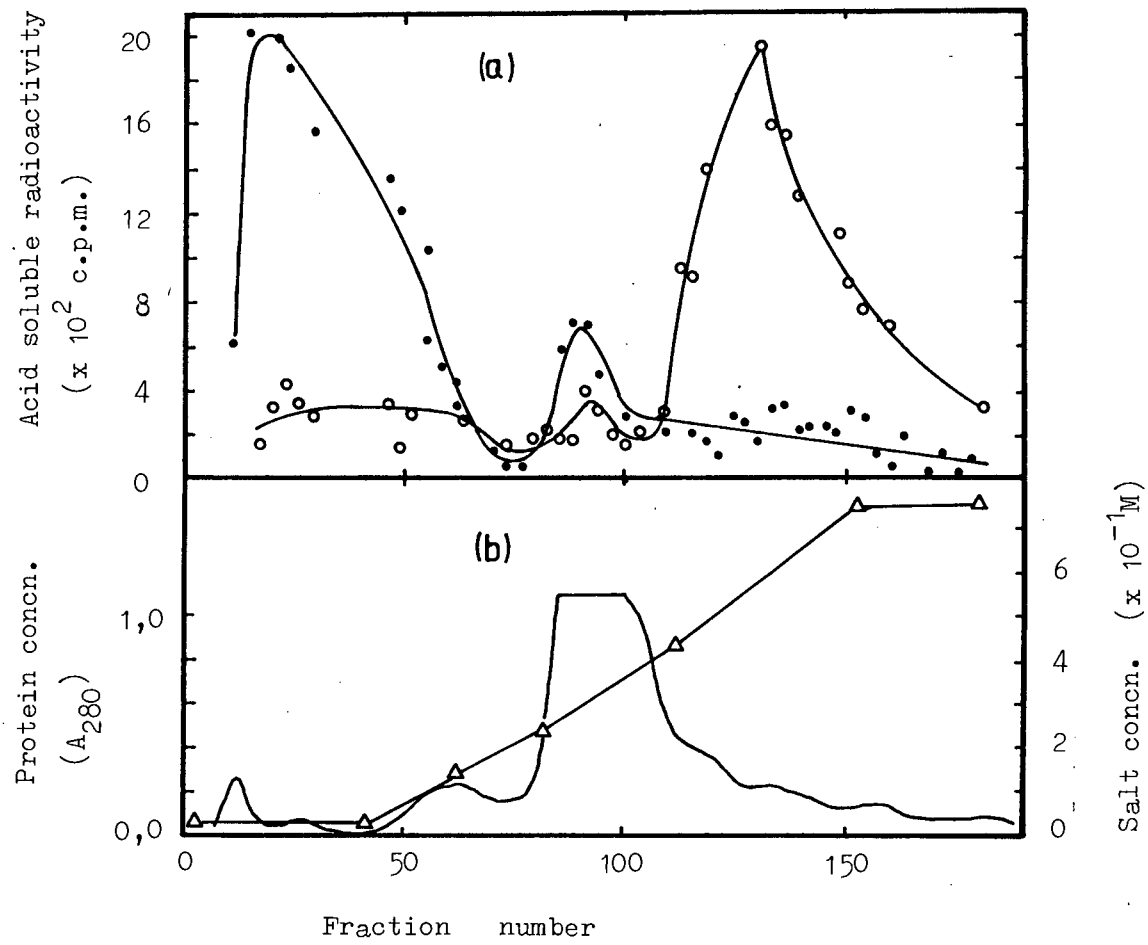


Fig. 4.3. Cation exchange chromatography of exponential phase Vibrio 'membrane' (P 100) preparation.

A sample (25 ml; ca. 2,1 g protein) of the preparation was loaded onto a 1,5 x 15 cm DE52 column; washed, and eluted with 800 ml of a 0 to 0,7 M NaCl gradient. Fractions (6 ml) were collected and assayed for (a) [^{14}C-me]globin degrading activity, with (o) and without (•) 3 mM ATP. Protein elution (A_{280}, followed using a recording spectrophotometer) and salt concentration (Δ) were followed (b). These data are from one of several independently repeated experiments, all showing similar results.

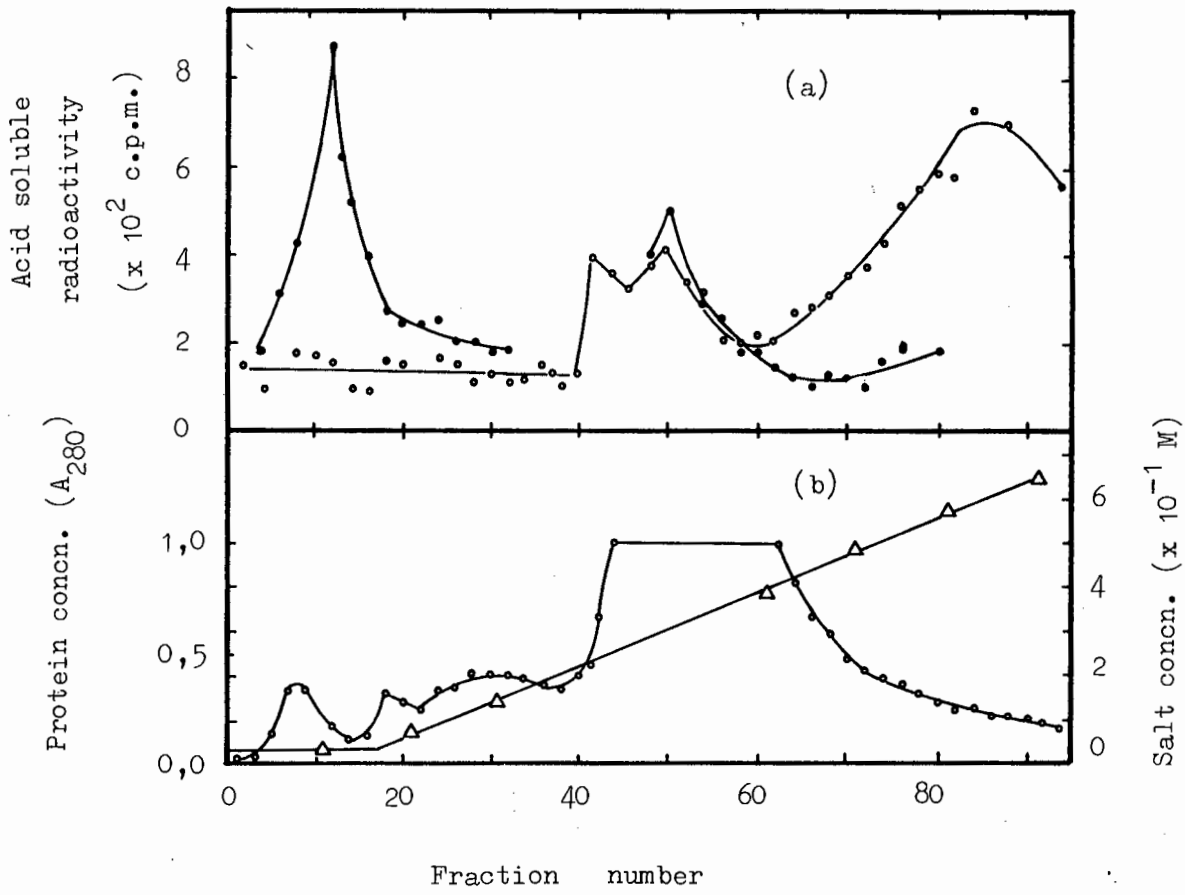


Fig. 4.4. Cation exchange chromatography of shaking stationary phase *Vibrio* 'membrane' (P 100) preparation.

A sample (20 ml; ca. 1,5 g protein) of the preparation was loaded onto a 1,5 x 15 cm DE52 column, washed, and eluted with 800 ml of a 0 to 0,7 m NaCl gradient. Fractions (6 ml) were collected and assayed for (a) [^{14}C-me]globin degrading activity with (o) and without (•) 3 mM ATP. Protein elution (A_{280}) and salt concentration (▲) were followed (b). These data are from one experiment. Similar experiments with standing stationary phase P 100 preparations gave similar results.

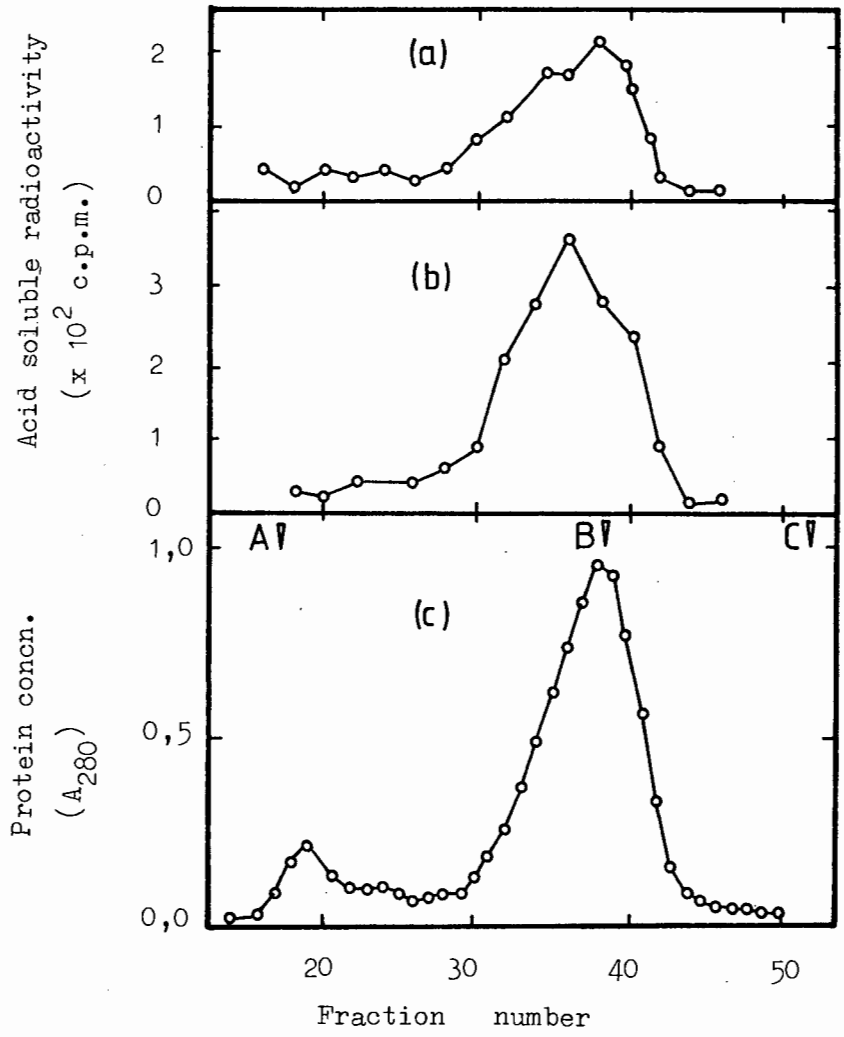


Fig. 4.5. Exclusion chromatography of exponential phase Vibrio cytoplasmic (S 100) preparation on Sephacryl S-1000.

Approximately 20 mg (1 ml) of protein was loaded onto a 0,9 x 60cm column, eluted with TM saline, and 0,85 ml fractions were collected. [^{125}I]-Insulin (a) and [^{14}C-me]globin (b) degrading activity was measured without ATP, as well as protein concentration (A_{280}; c). Markers used were (A) whole cells; (B) Haemoglobin; (C) phenol red. Repeat experiments gave identical results with both types of stationary phase Vibrio S 100 preparations.

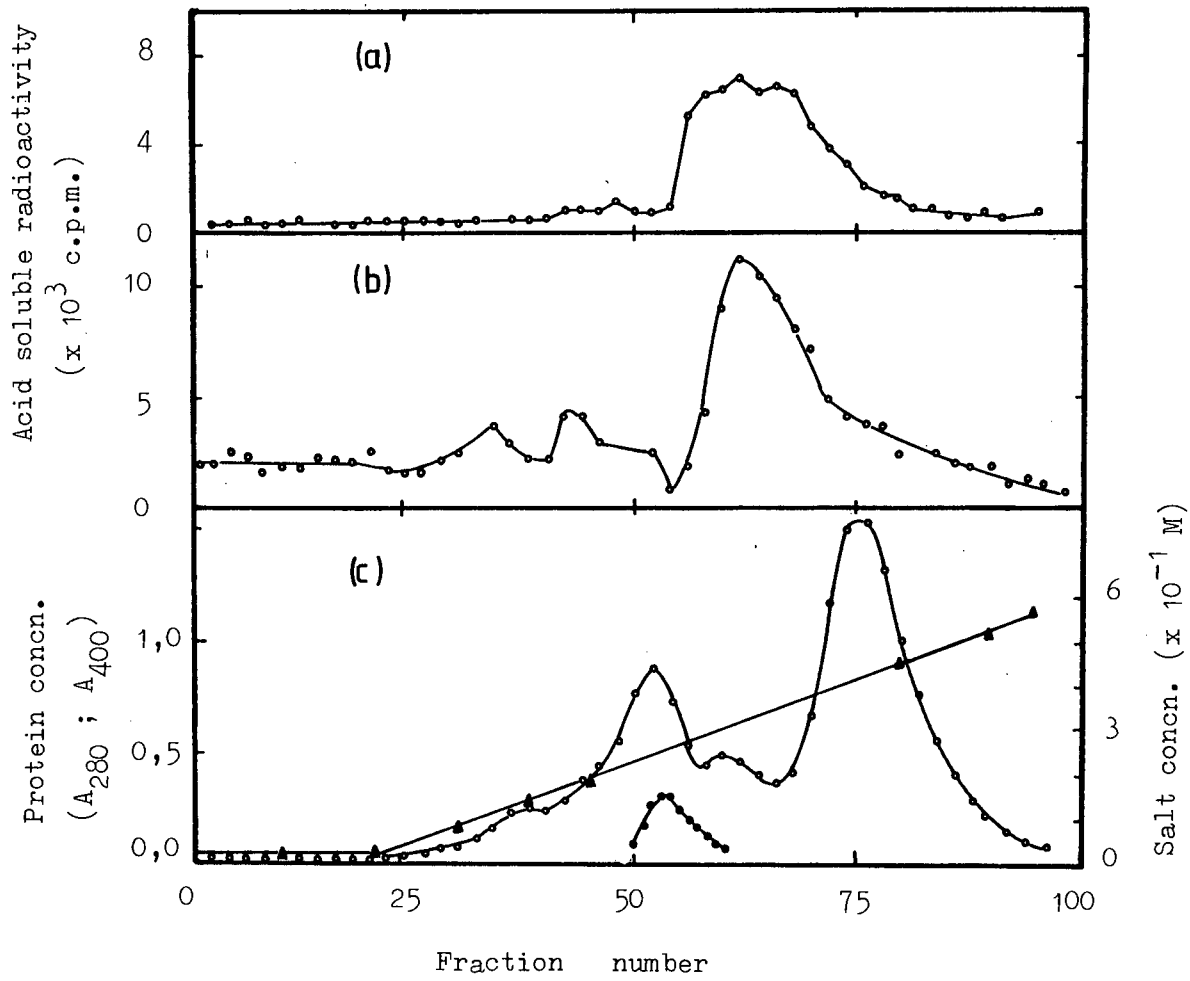


Fig. 4.6. Cation exchange chromatography of exponential phase Vibrio cytoplasmic (S 100) preparation.

A sample (25 ml; ca. 0,3 g protein) was loaded onto a 2,6 x 14 cm DE52 column, washed and eluted with 300 ml of a 0 to 0,5 M NaCl gradient. Fractions (4,3 ml) were collected and assayed for (a) [^{125}I]insulin and (b) [^{14}C-me]globin degrading activities. Globin degradation was also followed in the presence of 3 mM ATP, but did not differ significantly from that given (in the absence of ATP). Protein elution (A_{280}), salt concentration (▲) and the presence of a coloured protein (●; A_{400}) were followed (c). Fractions 52 to 75 were collected and treated as described (Figs. 4.9 & 4.10).

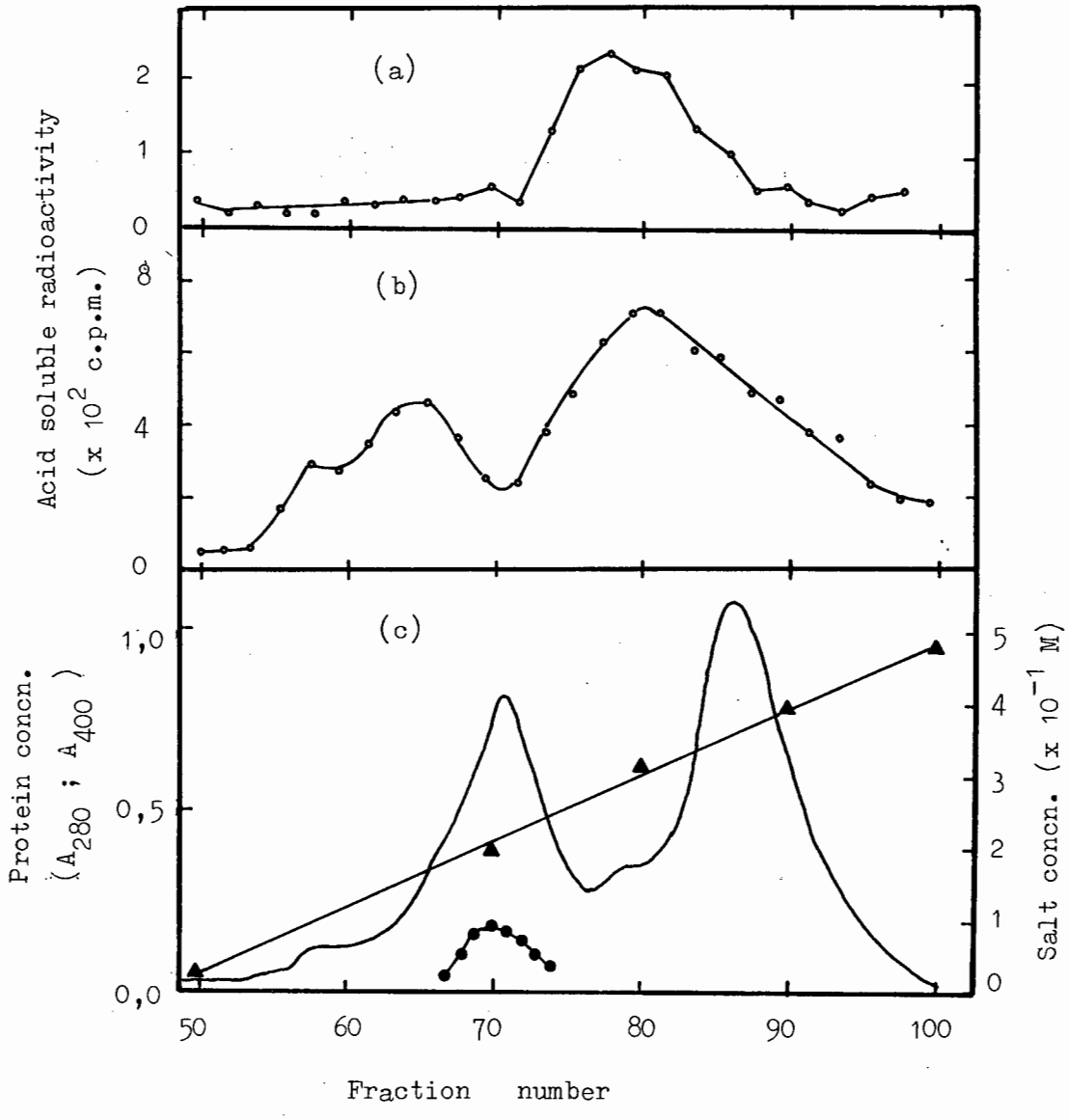


Fig. 4.7. Cation exchange chromatography of shaken stationary phase Vibrio cytoplasmic (S 100) preparation.

A sample (25 ml; ca. 0,2 g protein) was loaded onto a 2,6 x 14 cm DE52 column, washed and eluted with 300 ml of a 0 to 0,5 M NaCl gradient. Fractions (5,6 ml) were collected and assayed for (a) [^{125}I]-insulin and (b) [^{14}C-me]globin degrading activities. Globin degradation was also followed in the presence of 3 mM ATP, but did not differ significantly from that given (in the absence of ATP). Protein elution (A_{280}, solid curve, no symbols, followed using a recording spectrophotometer), salt concentration (▲) and the presence of a coloured protein (●; A_{400}) was followed (c).

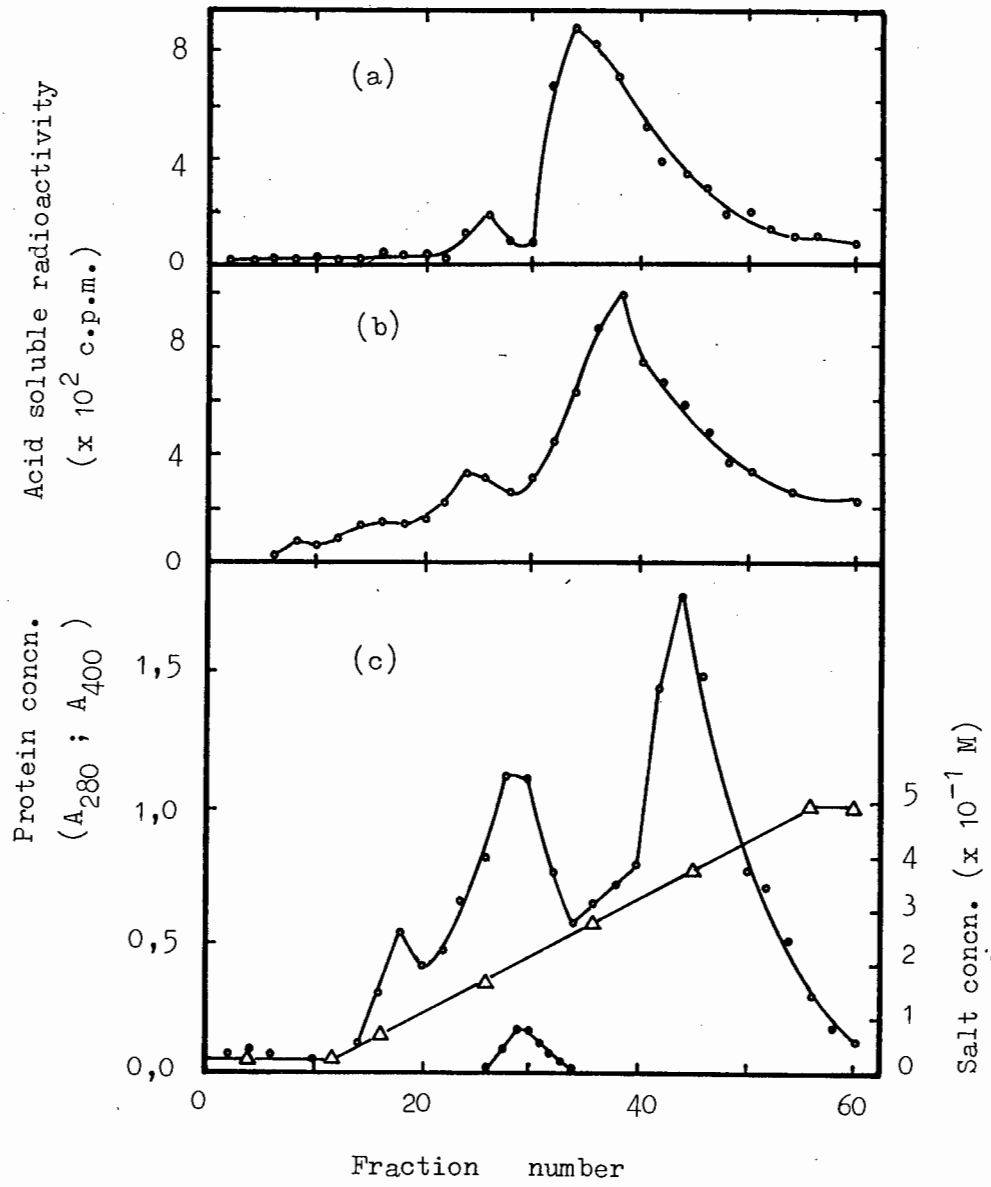


Fig. 4.8. Cation exchange chromatography of standing stationary phase *Vibrio* cytoplasmic (S 100) preparation.

A sample (25 ml; ca. 0,3 g protein) was loaded onto a 2,6 x 14 cm DE52 column, washed and eluted with 300 ml of a 0 to 0,5 M NaCl gradient. Fractions (5,6 ml) were collected and assayed for (a) [^{125}I]-insulin and (b) [^{14}C-me]globin degrading activities. Globin degradation was also followed in the presence of 3 mM ATP, but did not differ significantly from that given (in the absence of ATP). Protein elution (o;A_{280}), salt concentration (Δ) and the presence of a coloured protein (•; A_{400}) were followed (c).

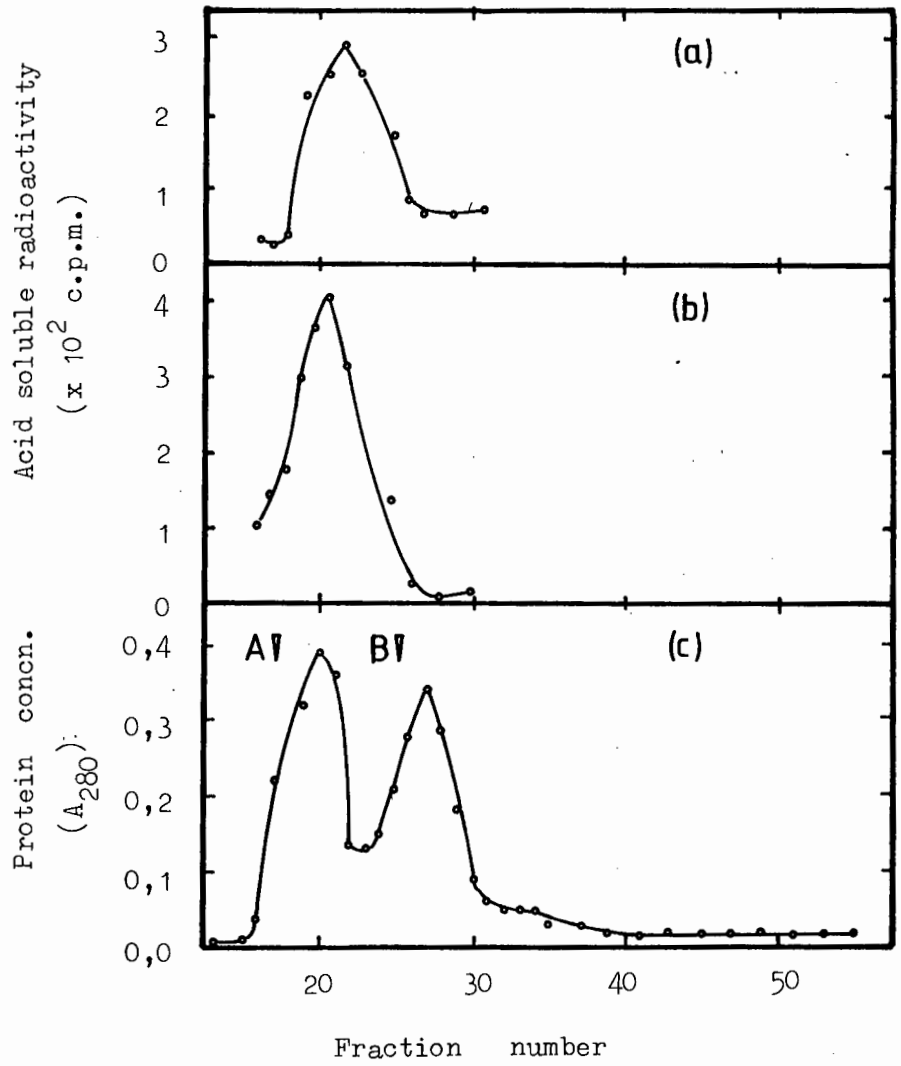


Fig. 4.9. Chromatography of the major peak from exponential phase Vibrio sp. 2 extracts, (Fig. 4.6) on Sephacryl S-200.

The pooled fractions (fractions 50-75, Fig. 4.6) were concentrated by 80 % ammonium sulphate precipitation, and a sample (ca. 1 ml) of the dialysed fraction (ca. 5 mg protein), was loaded onto a 0,9 x 60 cm column. Fractions (0,85 ml) were collected and assayed for (a) [^{125}I]-insulin and (b) [^{14}C-me]globin degrading activities, as well as protein concentration (c). The proteolytic activities reproducibly eluted between the void volume (A) and the haemoglobin marker (B). Similar results were obtained with both types of stationary phase cells.

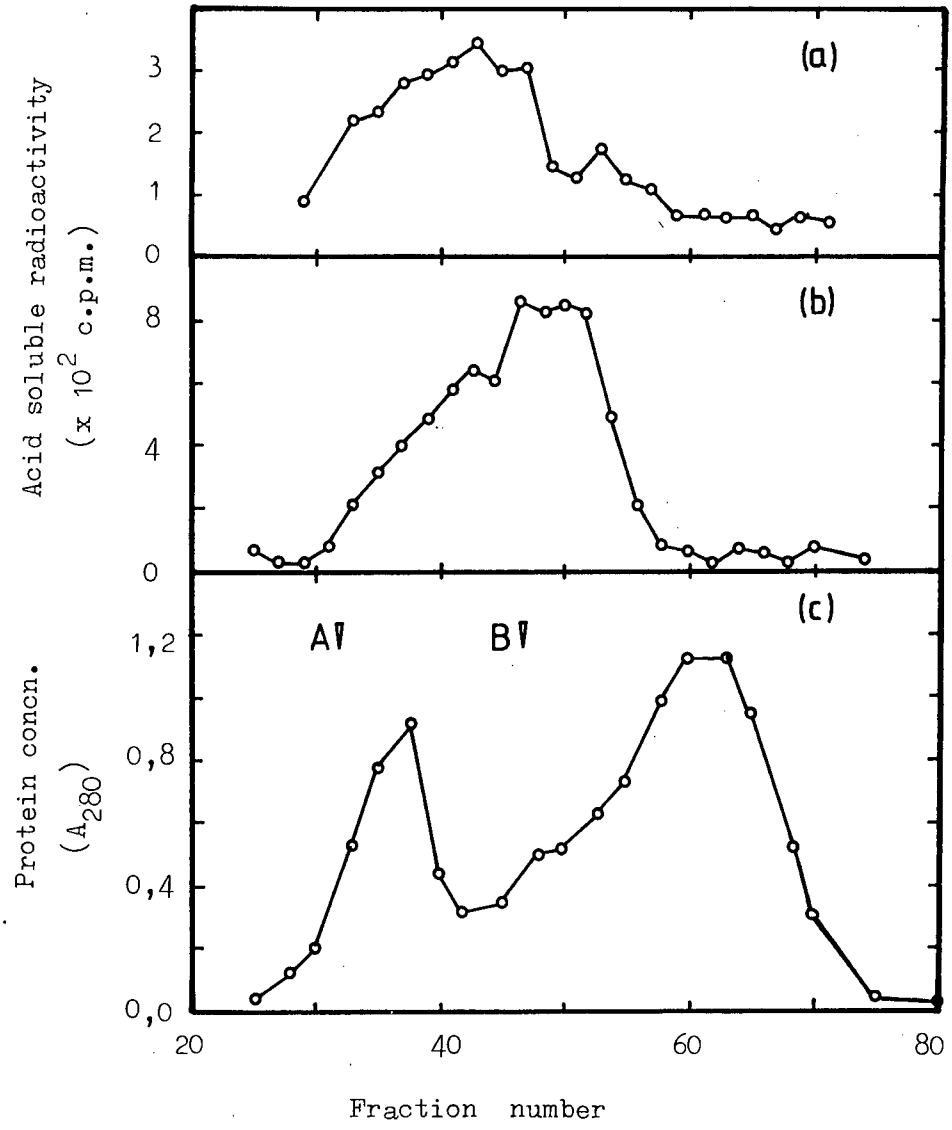


Fig. 4.10. Exclusion chromatography of the major proteolytic activity peak from exponential phase Vibrio preparations (Fig. 4.6) on Sephacryl S-200.

The pooled fractions (fractions 50-75, Fig. 4.6) were concentrated by ultrafiltration (to ca. 5 ml), and loaded onto a 1,6 x 80 cm column. Fractions (2 ml) were collected and assayed for both (a) [^{125}I]-insulin and (b) [^{14}C-me]globin degrading activities. Protein elution (c) was followed. Markers used were ferritin [M_r 450 000; (A)] and bovine serum albumin [M_r 67 000; (B)].

Cation-exchange chromatography of exponential phase Vibrio membrane preparation (P 100) separated 3 distinct [^{14}C-me]globin degrading activities (Fig. 4.3), one activity, which was markedly inhibited by the presence of 3 mM ATP, eluted before the salt gradient was applied. A second activity eluted at 0,3 M NaCl, and a third activity, active only in the presence of ATP, eluted at 0,6M NaCl. This same pattern was found for exponential and both types of stationary phase extracts, (Figs. 4.3 & 4.4; results for shaking stationary phase extracts only are given). In all cases there was negligible [^{125}I]-insulin degrading activity observed.

4.3.3. Cytoplasmic proteolytic activity.

A membrane-free exponential phase cytoplasmic preparation (S 100) subjected to exclusion chromatography (Fig. 4.5) on Sephacryl S-1000 showed the elution of globin and insulin degrading activities, as well as a large portion of the Vibrio soluble protein, near the haemoglobin standard.

Membrane-free cytoplasmic preparations (S 100) subjected to cation-exchange chromatography (Figs. 4.6; 4.7 and 4.8) showed similar patterns of protein elution and proteolytic activity in both exponential and both types of stationary phase preparations. All preparations showed one major [^{125}I]-insulin degrading activity, which eluted at approximately 0,3 M NaCl. The standing stationary phase S 100 extract had a minor activity which eluted at 0,15 M NaCl (Fig. 4.8). Both stationary phase extracts had two separable [^{14}C-me]globin degrading activities which eluted at 0,15 M NaCl (minor activity) and 0,3 M NaCl

(major activity; Fig. 4.7 & 4.8). In addition to the major globin degrading activity, exponential phase Vibrio S-100 extract had two minor activities which eluted at 0,1 M and 0,15 M NaCl (Fig. 4.6). In all cases, globin degradation was also measured in the presence of 3 mM ATP, which was found not to influence globin degradation significantly (within 15 %) compared to globin degradation in the absence of ATP.

Both globin and insulin degrading activities eluted at approximately 0,3 M NaCl in all cases. When the fractions 52 to 75 from exponential phase cytoplasmic preparations were pooled (Fig. 4.6), concentrated by precipitation with 80 % ammonium sulphate, and chromatographed on a Sephacryl S-200 (exclusion limit 250 000) column (Fig. 4.9), the activities co-eluted between the void volume and the haemoglobin marker. However, when the pool of fractions was concentrated by ultrafiltration (Fig. 4.10) the activities were eluted separately, close to the BSA standard.

4.4. Discussion.

Vibrio sp. 2 has distinct ATP-affected proteolytic activities, an ATP-stimulated and an ATP-depressed activity, separable by ion-exchange chromatography (Figs. 4.3 & 4.4). These activities appear to be membrane associated, in that they were detected in membrane enriched (P 100) fractions, but were not detected in S 100 preparations (Figs. 4.6; 4.7 & 4.8). Furthermore, the ATP-depressed proteolytic activity eluted with the large particulate fraction upon molecular exclusion chromatography of an exponential phase whole cell extract (Fig. 4.1), and during isopycnic centrifugation, settled in a band of density 1,23 g ml^{-1}

(Table 4.3). It cannot, however, be unequivocally stated that the activity referred to is outer-membrane associated, as the purity of the putative inner and outer membrane preparations could not be ascertained (4.3.2).

There are a variety of membrane associated proteolytic activities in E. coli, both in the inner and outer membranes. Voellmy & Goldberg (1981) demonstrated an ATP-stimulated proteolytic activity associated with the membrane, while other workers have shown serine proteases present in the E. coli outer membrane (1.5.9). The activities of these latter serine proteases were not affected by ATP.

The complement of proteolytic activities in exponential and both types of stationary phase Vibrio cells appears to be generally similar, although there were some differences. The fractions P 100 and S 100 taken together are assumed to represent the whole cell extract including the periplasmic contents, as the cells were not osmotically shocked prior to lysis. Vibrio cells had a single insulin-degrading peak of activity [Fig. 4.6 (a); 4.7 (a) & 4.8 (a)] although standing stationary phase extracts had a second insulin-degrading activity [Fig. 4.8 (a)]. Similarly, Vibrio cell-free extracts, both membrane enriched (P 100) and membrane free (S 100), had similar complements of globin degrading activities. The number of globin degrading activities cannot be unequivocally stated, as firstly the centrifugation protocol used to prepare the P 100 and S 100 fractions may not have entirely removed cytoplasmic activities from the membrane activities, and vice versa, and secondly the peaks themselves may not have represented single proteolytic species. In the separation of proteolytic activities from E. coli extracts by ion-exchange chromatography, five peaks of

activities were obtained, one of which, peak 1, yielded two activities after cation-exchange chromatography, and another peak, peak 2, yielded three distinct activities after anion-exchange chromatography (Goldberg et al., 1981). Pooled Vibrio proteolytic peak fractions obtained after cation-exchange chromatography were analysed by anion-exchange chromatography (on carboxymethyl-cellulose), but without success. The problems encountered were the lack of sufficient activity within the pooled fractions for further analysis, and the loss of the effect ATP addition had on the proteolytic activities within 24 h after collection of the fractions. Analysis of E. coli proteolytic activities required quantities of cell-free extract which could not be produced in this study.

Fractions containing the ATP-stimulated membrane-associated E. coli proteolytic activity hydrolysed [^{35}S]-labelled E. coli proteins to TCA soluble material of heterogeneous size (Voellmy & Goldberg, 1981), whereas the proteolytic activities in whole cell E. coli extracts hydrolysed the same substrate to amino acids within the same period. In this study with Vibrio sp. 2, band 3 (Table 4.3), assumed to represent membrane material (a putative mixture of unseparated inner and outer membranes) hydrolysed [^{14}C-me]globin to TCA soluble material with molecular masses in the range 6 000 daltons to several hundred daltons [Fig. 4.2 (b)] within 1 h, however on prolonged incubation (16 h), the substrate was all hydrolysed to material of several hundred daltons [Fig. 4.2 (c)].

It has been proposed (St. John & Goldberg, 1976) that proteins in a bacterial cell are degraded initially by an energy requiring endoproteolytic event followed by the hydrolysis of the products by a

variety of proteases and peptidases. The initial endoproteolytic event could occur near the cytoplasmic membrane (1.5.3), and soluble cytoplasmic enzymes (exo- and endopeptidases) could catalyse the subsequent conversion of the peptides to free amino acids. This postulated localisation of proteolytic activities might affect the selectivity of protein breakdown, as proteins with abnormal conformations may have more hydrophobic regions exposed on their surfaces than normal proteins (St. John & Goldberg, 1976) and may differentially bind to the cell membrane. When bacteria make large amounts of abnormal protein, these molecules aggregate, from granules adjacent to the cytoplasmic membrane, and are subsequently found as fragments in the cytoplasm (1.5.3).

Molecular masses of the E. coli intracellular proteases range from a large mass of 520 000 daltons for protease Do, as determined by molecular exclusion chromatography, to masses of 140 000 daltons for protease So (Chung & Goldberg, 1983) and 94 000 daltons for protease La (Charette et al., 1981). Although none of the proteolytic activities of Vibrio sp. 2 has been purified to homogeneity, an indication of the sizes of the major proteolytic activities was obtained by molecular exclusion chromatography. The major proteolytic activities in the S 100 preparation of the exponential and both types of stationary phase cells eluted near the haemoglobin marker (Mr 65 000) as shown in Fig. 4.5, for both the insulin and globin degrading activities. These activities obtained as the pooled fractions after cation exchange chromatography (Fig. 4.6), concentrated by ammonium sulphate precipitation eluted reproducibly between the void volume and the haemoglobin marker on molecular exclusion chromatography on Sephacryl S-200, and after concentration by ultrafiltration, the globin degrading activity eluted

near the BSA marker (Mr 67 000) and the insulin-degrading activity eluted between the ferritin and BSA markers.

Experiments of this type with the separated ATP affected proteolytic activities did not yield meaningful results due to the low level of activity obtained after such analysis. The possibility that the effect of ATP on Vibrio proteolytic activities may be mediated by some membrane-related function, such as membrane bound ATP-ases, or protein kinases cannot be ruled out by the studies presented here. Such unequivocal demonstration would require the purification to homogeneity of the ATP affected activities, such as has been achieved with protease La from E. coli.

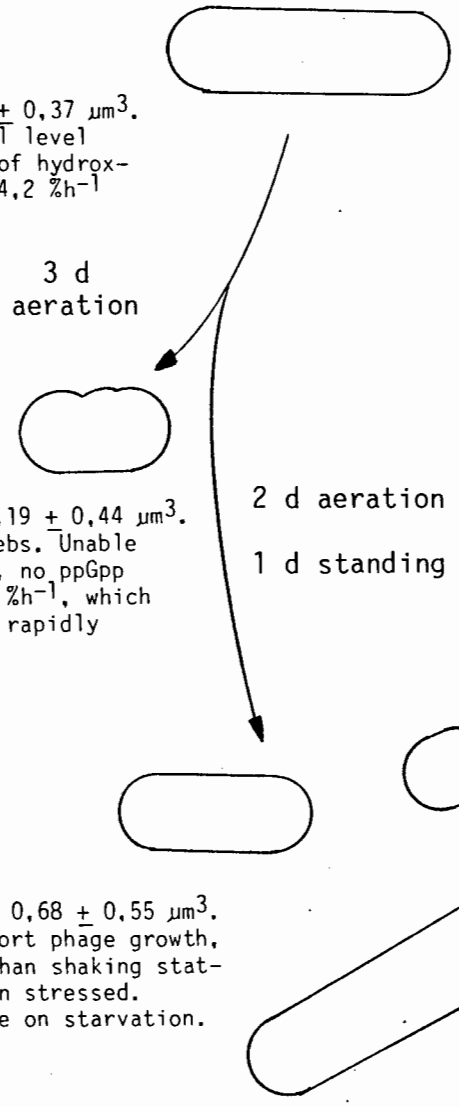
CONCLUSION

The model of Vibrio sp. 2 development during growth and existence in stationary phase (Fig. 2.22) has been extended here by including data presented in this thesis (Fig. 5.1). Although the changes that occurred in Vibrio sp. 2 differentiation are not as dramatic as those that occur during the sporulation process in bacteria, nevertheless, changes did occur that enabled non-growing Vibrio sp. 2 to support phage Alpha 3a growth. Since the concentration of protein in standing and shaking cultures were identical (Robb, 1980), the morphological changes indicate a reorganisation of the available cellular components. Stationary phase bacterial cells are capable of adapting to environmental changes at the transcriptional level (Mandelstam, 1957) and the ability of cells to degrade and reutilise existing macromolecules is of importance when the re-synthesised molecules differ from those degraded, a process that has been referred to as biochemical differentiation (Mandelstam, 1960).

The widely accepted view of bacterial life is generally derived from studies of mesophiles such as E. coli, such that protein turnover in growing bacteria is generally held to be low, for example 1 - 2 $\%h^{-1}$ in E. coli (Nath & Kock, 1970; Pine, 1972; Goldberg & St. John, 1976), although some specific proteins such as l-serine deaminase have been shown to be rapidly turned over during growth (Beeraj et al., 1978). In stationary phase, under nutritional shift-down conditions, amino-acid starvation, or other unfavourable conditions, the rate of protein turnover can increase to 5 - 12 $\%h^{-1}$ in mesophiles (Goldberg & St. John,

Exponential phase cell

Motile cells, 3,16 ± 0,81 μm long of volume 1,12 ± 0,37 μm^3. Presence of blebs. Supports Alpha 3a growth, basal level of ppGpp detectable, which increases with stress of hydroxylamine. Protein turnover 1,8 $\%h^{-1}$, increases to 4,2 $\%h^{-1}$ on starvation



Shaking stationary phase cell

Non-motile cells, 2,11 ± 0,45 μm long of volume 1,19 ± 0,44 μm^3. Cell wall often with indentations, seldom with blebs. Unable to support phage growth, low ATP content per cell, no ppGpp detectable, even when stressed. Protein turnover 1 $\%h^{-1}$, which increases to 3 $\%h^{-1}$ on starvation, has a class of rapidly turned-over proteins.

Standing stationary phase cell

Motile cells, 2,01 ± 0,87 μm long, with volume of 0,68 ± 0,55 μm^3. Coccoid and elongated cells present. Able to support phage growth, more resistant to u.v. and thermal inactivation than shaking stationary phase cells, no ppGpp detectable, even when stressed. Protein turnover 2,9 $\%h^{-1}$, not reliably measurable on starvation.

Vibrio sp.2 cells all possess separate ATP-stimulated and depressed proteolytic activities, as well as several proteolytic activities not affected by ATP. Although the complement of proteolytic activities in the cells is similar, there are differences in the subcellular distribution of proteolytic activities.

Fig. 5,1. Model of Vibrio sp.2 stationary phase phenomena.

This diagram depicts the model of Vibrio sp.2 development including information presented in this thesis.

1976). It has been suggested (Pine, 1970; Wheatley et al., 1978) that the increased protein turnover is due to the abnormal state of the proteins themselves, rather than the formation of new proteases, although sporulating bacteria do produce new proteases during sporulation (1.5.3).

In E. coli, acidic proteins are degraded more rapidly than are neutral or basic proteins (Dice & Goldberg, 1975). It has been shown that proteins of extreme halophiles, and to a lesser extent, of moderate halophiles are more acidic than those of non-halophilic bacteria (Kushner, 1978), and Hipkiss et al., (1980) have shown that protein turnover in the moderate halophile Vibrio costicola is substantially greater than that in E. coli. Vibrio sp. 2, which is halotolerant, that is, does not require salt for growth, but is able to grow in salt concentrations of 10 % or more (Thompson, 1973), has a relatively low protein turnover rate of 1,8 $\%h^{-1}$ (Ch. 1) in exponentially growing cells, and rates of between 1 and 3 $\%h^{-1}$ in stationary phase, depending on the culture conditions. There is also a class or rapidly turned over proteins in shaken stationary phase cells as shown by pulse labelling experiments.

The lack of detectable magic spot nucleotides in stationary phase Vibrio sp. 2 cells suggests that these nucleotides, though evident in stressed exponential phase Vibrio sp. 2 cells, and shown to be involved in regulatory phenomena in slowly growing E. coli cells (1.4.1), are not involved in developments occurring in stationary phase Vibrio sp. 2.

The degradation of the substrates [^{14}C-me]globin and [^{125}I]-insulin by cell-free Vibrio sp. 2 extracts shows that although the complement of proteolytic activities were similar in the three types of cultures (Ch. 4), the proteolytic activity of extracts from exponential and both types of stationary phase cells differed with regard to specific activity, subcellular distribution and response to the presence of protease inhibitors or the addition of ATP.

APPENDIX

Broths and buffers.

Tryptone broth.

Tris/HCl (100 mM;pH 7,6), tryptone (13,0 g/l), NaCl (0,4 M), glucose (1,5 g/l)

The ingredients were dissolved, distributed as required and autoclaved in standard conditions. For solid medium, 15 g/l of Difco agar was added before autoclaving.

TEAS buffer.

Triethanolamine (50 mM; pH 7,6), sucrose (10 % w/v).

Immediately prior to use, 0,1 ml of a freshly prepared lysozyme stock solution (10 mg ml^{-1}) was added to 10 ml buffer.

TMKD buffer

Tris/HCl (50 mM; pH 8,0), $MgCl_2$ (10 mM), KCl (10 mM), dithiotrietol (0,5 mM)

The buffer was made up and stored without dithiothrietol, at 4 ^{o}C. The dithiothreitol was added immediately prior to use.

TES buffer

Tris/HCl (30 mM; pH 8,0), sucrose (20 % w/v), EDTA (sodium salt; 10 mM), lysozyme (100 µg/ml).

Sodium EDTA (pH 8,0) was added to the buffer at a final conc. of 10 mM. Lysozyme (100 µg ml^{-1}) was added from a 10 mg ml^{-1} stock solution immediately prior to use.

TSMMKD buffer

Triethanolamine (50 mM; pH 8,0), sucrose (10 % w/v), $MgSO_4.7H_2O$ (5 mM), KCl (0,5 M).

The buffer was made up and autoclaved. Immediately prior to use, 50 ul of a 1 mg ml^{-1} stock solution of protease free DNase was added per 10 ml buffer.

TM buffer

Tris/HCl (10 mM; pH 7,6), $MgCl_2.6H_2O$ (10 mM).

The buffer was made up and stored at 4 ^oC. Variations of this buffer were:

TM saline (150 mM NaCl in TM buffer)
TMM buffer (10 mM 2-ME in TM buffer)
TMM saline (10 mM 2-ME in TM saline)
TMK buffer (50 mM KCl in TM buffer)

Liquid Scintillation Counting

Radioactivity was quantified by conventional liquid scintillation counting in a Packard scintillation counter. Beckmann 'Ready-solve EP' premixed scintillation cocktail was used according to the manufacturer's recommendations. Radioactivity of the [^{125}I]-insulin was determined in a Packard Tri-carb gamma counter.

BIBLIOGRAPHY

Adams, M.H. (1959).
'The Bacteriophages'
New York, Interscience Publishers.

Amy, P.S., Pauling, C., Morita, R.Y. (1983 a).
Starvation-survival process of a marine Vibrio.
App. Env. Microbiol. 45;1041-1048.

Amy, P.S., Pauling, C., Morita, R.Y. (1983 b).
Recovery from nutrient starvation by a marine Vibrio sp.
App. Env. Microbiol. 45;1685-1690.

Amy, P.S., Morita, R.Y. (1983).
Protein patterns of growing and starved cells of a marine Vibrio sp.
App. Env. Microbiol. 45;1748-1752.

Arbige, M., Chesbro, W. (1982 a).
Rel A and related loci are growth rate determinants for Escherichia coli in a recycling fermenter.
J. Gen. Microbiol. 128;693-703.

Arbige, M., Chesbro, W.R. (1982 b).
Very slow growth of Bacillus polymyxa: stringent response and maintenance energy.
Arch. Microbiol. 132;338-344.

Asakura, T. (1978).
Haemoglobin porphyrin modifications.
in 'Methods in Enzymology' LII;447-449.
Eds. Fleisher, S. Packer, L. Academic Press, New York.

Ayukawa, S., Fisher, J.M., Rabinovitz, M. (1981).
Proteolytic susceptibility of hemoglobin synthesised in the presence of amino acid analogues.
Molec. Pharmacol. 20;227-229.

Baker, D.A., Park, R.W.A. (1975).
Changes in morphology and cell wall structure that occur during growth of Vibrio sp. NCTC 4716 in batch culture.
J. Gen. Microbiol. 86;12-28.

Beeraj, R., Morris, J. F., Newman, E.B. (1978).
Factors influencing the in vivo stability of l-serine deaminase stability in E. coli .
Can. J. Microbiol. 24;1607-1613.

Bennett, J.P. (1982).
Solubilisation of membrane-bound enzymes and analyses of membrane protein concentrations.
In 'Techniques in Lipid and Membrane Biochemistry.' B408;1-22.
Elsevier/North Holland Scientific Publishers, County Clare, Ireland

Bernlohr R.W., Clarke, V. (1971).
Characterisation and regulation of protease synthesis and activity in Bacillus licheniformis.
J. Bacteriol. 105;276-283.

Blobel, G., Dobberstein, B. (1975).
Transfer of proteins across membranes. 1. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine melanoma.
J. Cell Biol. 67;835-851.

Block, R., Haseltine, W.A. (1974).
In vitro synthesis of ppGpp and pppGpp.
In 'Ribosomes', Ed. Nomura, M., Tissieres, A. and Lengyel, P.
Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y. pp. 733-745.

Boches, F.S., Klemes, Y., Goldberg, A.L. (1980).
Purification of a non-lysosomal, ATP-stimulated protease from rabbit reticulocytes.
Fed. Proc. Fed. Am. Soc. Exp. Biol. 39;1682.

Bowles, L.K., Konisky, J. (1981).
Cleavage of colicin Ia by the Escherichia coli K12 outer membrane is not mediated by Ia receptor.
J. Bacteriol. 145;668-671.

Boylen, C.W., Ensign, J.C. (1970).
Long term starvation survival of rod and spherical cells of Arthrobacter crystallopoeties.
J. Bacteriol. 103;569-577.

Brey, R.N. (1982).
Fragmentation of colicins A and E1 by cell surface proteases.
J. Bacteriol. 149;306-315.

Brunschede, H., Bremer, H. (1971).
Synthesis and breakdown of proteins in Escherichia coli during amino-acid starvation.
J. Mol. Biol. 57;35-57.

Bukhari, A.I., Zipser, D. (1973).
Mutants of Escherichia coli with a defect in the degradation of nonsense fragments.
Nature New Biol. 243;238-241.

Car, N.G., Woods, D.R. (1984).
Protein degradation in extracts of exponential and stationary phase Vibrio cells.
J. Gen. Microbiol. 130;2775-2781.

Cashel, M., Gallant, J. (1969).
Two compounds implicated in the function of the RC gene of Escherichia coli.
Nature 221;838-841.

Cashel, M. (1975).
Regulations of bacterial ppGpp and pppGpp.
Annu. Rev. Microbiol. 29;301-318.

Cavard, D., Pages, J.M., Lazdunski, C.J. (1982).
A protease as a possible sensor of environmental conditions in E.coli outer membrane.
Mol. Gen. Genet. 188;508-512.

Cavard, D., Lazdunski, C. (1979).
Interaction of colicin E4 with specific receptor sites mediates its cleavage into two fragments inactive towards whole cells.
Eur. J. Biochem. 96;525-533.

Chaloupka, J., Strnadova, M. (1980).
Degradation and replenishment of the labile protein fraction in non-growing cells of the asporogenic mutant of Bacillus megaterium.
Folia Microbiologica 25;191-200.

Chaloupka, J., Strnadova, M. (1982).
Kinetics of protein turnover in growing cells of Bacillus megaterium.
J. Gen. Microbiol. 128;1003-1008.

Chang, C.N., Model, P., Inouye, H., Beckwith, J. (1980).
Alkaline phosphate precursor is processed to the mature enzyme by an E.coli inner membrane preparation.
J. Bacteriol. 142;726-728.

Charette, M.F., Henderson, G.W. Markovitz, A. (1981).
ATP hydrolysis-dependent protease activity of the lon (capR) protein of Escherichia coli K12.
Proc. Natl. Acad. Sci. U.S.A. 78;4728-4732.

Cheng, Y.E., Zipser, D., Cheng, C.Y., Rolseth, S.J. (1979).
Isolation and characterization of mutations in the structural gene for protease III (ptr).
J. Bacteriol. 140;125-130.

Cheng, Y.E., Aronson,A.I. (1977).
Alteration of spore coat processing and protein turnover in a Bacillus cereus mutant with a defective post exponential intracellular protease.
Proc. Natl. Acad. Sci. U.S.A. 74;1254-1258.

Cheng, Y.E., Zipser, D. (1979).
Purification and characterisation of protease III from Escherichia coli.
J. Biol. Chem. 254;4698-4706.

Chesbro, W., Evans, T., Eifert, R. (1979).
Very slow growth of Escherichia coli.
J.Bacteriol. 139;625-638.

Chung, C. H-. Goldberg, A.L. (1982).
DNA stimulates ATP-dependent proteolysis and protein-dependent ATPase activity of protease La from Escherichia coli.
Proc. Natl. Acad. Sci. U.S.A. 79;795-799.

Chung, C.H., Goldberg, A.L. (1982).
DNA stimulates ATP-dependent proteolysis and protein-dependent ATP-ase activity of protease La from Escherichia coli.
Proc. Natl. Acad. Sci. U.S.A. 79;795-799.

Cohen, S.S., Arbogast, R. (1950).
Chemical studies in host-virus interactions VII. A comparison of some properties of three mutant pairs of bacterial viruses T2r$^+$ and T2r, T4r$^+$, T6r$^+$ and T6r.
J. Exp. Med. 91;619-636.

Craig, N.L., Roberts, J.W. (1980).
E.coli Rec A protein-directed cleavage of phage lambda repressor requires polynucleotide.
Nature 283;26-29.

Davis, B.D., Tai, P.-C. (1980).
The mechanism of protein secretion across membranes.
Nature 283;433-438.

Dean, A.C.R., Henselwood, C. (1966).
"Growth, function and regulation in bacterial cells."
London. Oxford University Press.

Dice, J.F., Goldberg, A.L. (1975).
Relationship between in vivo degradative rates and isoelectric points of proteins.
Proc. Natl. Acad. Sci. U.S.A. 72;3893-3897.

DiRienzo, J.M., Nakamura, K., Inouye, M. (1978).
The outer membrane proteins of gram-negative bacteria: biosynthesis, assembly, and functions.
Annu. Rev. Biochem. 47;481-532.

Dix, D.B., Thompson, R.C., Mackow, E.R., Chang, F.N. (1983).
Effect of ppGpp on the accuracy of protein biosynthesis.
Arch. Biochem. Biophys. 223;319-324.

Dixon, M. (1953).
A nonogram for ammonium sulphate solutions.
Biochem. J. 54;457-458.

Doi, R.H. (1972).
Role of proteases in sporulation.
Curr. Top. Cell Regulat. 6;1-20.

Dykstra, C., Kushner, S.R. (1985).
Physical characterisation of the cloned protease III gene from Escherichia coli K12.
J. Bacteriol. 163;1055-1059.

Edelman, P., Gallant, J. (1977).
Mistranslation in E.coli.
Cell 10;131-137.

Ensign, J.C. (1970).
Long term starvation survival of rod and spherical cells of Arthrobacter crystallopoietes.
J. Bacteriol. 103;569-577.

Epstein, I., Grossowicz, N. (1969).
Intracellular protein breakdown in a thermophile.
J. Bacteriol. 99;418-421.

Etlinger, J.D., Goldberg, A.L. (1977).
A soluble ATP-dependent proteolytic system responsible for the degradation of abnormal proteins in reticulocytes.
Proc. Natl. Acad. Sci. U.S.A. 74;54-58.

Fiil, N.P., Willumsen, B.M., Friesen, J.D., Von Meyenburg, K. (1977).
Interaction of alleles of the relA, relC and spoT genes in Escherichia coli: analysis of the interconversion of GTP, ppGpp and pppGpp.
Mol. Gen. Genet. 150;87-101.

Finley, D., Varshavsky, A. (1985).
The ubiquitin system: functions and mechanisms.
Trends Biochem. Sci. 10;343-347.

Foley, D., Dennis, P., Gallant, J. (1981).
Mechanism of the rel defect in beta-galactosidase synthesis.
J. Bacteriol. 145;641-643.

Gallant, J.A. (1979).
Stringent control in E. coli.
Annu. Rev. Genet. 13;393-415.

Gallant, J., Lazzarini, R. (1976).
The regulation of ribosomal RNA synthesis and degradation in bacteria.
In 'Protein Synthesis: A Series of Advances.' ed. Mc Conkey, E.
Dekker, New York. pp. 309-359.

Gallant, J., Shell, L., Bittner, R. (1976).
A novel nucleotide implicated in the response of Escherichia coli to energy source downshift.
Cell 7;75-85.

Gausing, K. (1980).
In 'Ribosomes: Structure, Function and Genetics' Eds. Chambliss, G.,Craven, G.R., Davies, J., Davis, K., Kahan, L. and Nomura, M.
Univ. Park. Baltimore, U.S.A. pp. 693-718.

Geesey, G.G., Morita, R.Y. (1979).
Capture of arginine at low concentrations by a marine psychrophilic bacterium.
Appl. Env. Microbiol. 38;1092-1097.

Goldberg, A.L. (1972 a).
Correlation between rates of degradation of bacterial proteins in vivo and their sensitivity to proteases.
Proc. Natl. Acad. Sci. U.S.A. 2640-2644.

Goldberg, A.L. (1972 b).
Degradation of abnormal proteins in Escherichia coli.
Proc. Natl. Acad. Sci. U.S.A. 69;422-426.

Goldberg, A.L., St John, A.C. (1976).
Intracellular protein degradation in mammalian and bacterial cells : Part 2.
Annu. Rev. Biochem. 44;747-803.

Goldberg, A.L., Dice, J.F. (1974).
Intracellular protein degradation in mammalian and bacterial cells.
Annu. Rev. Biochem. 43;835-869.

Goldberg, A.L., Swamy, K.H.S., Chung, C.H., Larimore, F.S. (1981).
Proteases in Escherichia coli.
Methods in Enzymol. Ed. Lorand, L.
Academic Press, New York, 80;680-702.

Goldberg, A.L. (1971).
Nature New Biol. 234;51-52.

Goldschmidt, R. (1970).
In vivo degradation of nonsense fragments in E.coli.
Nature 228;1151-1154.

Groat, R.G. Matin, A. (1986).
Synthesis of unique proteins at the onset of carbon starvation in Escherichia coli.
J. Indust. Microbiol. 1;69-73.

Hall, B.G., Gallant, J. (1972).
Defective translation in RC$^-$ cells.
Nature New Biol. 237;131-135.

Hall, M.N., Silhavy, T.T. (1981 a).
The ompB locus and the regulation of the outer membrane porin proteins of Escherichia coli K-12.
J. Mol. Biol. 146;23-43.

Hall, M.N., Silhavy, T.T. (1981 b).
Genetic analysis of the ompB locus in Escherichia coli K-12
J. Mol. Biol. 151;1-15.

Haseltine, W.A., Block, R., Gilbert, W., Weber, K. (1972).
MSI and MSII made on ribosome in idling step of protein synthesis.
Nature 238;381-384.

Hayes, W. (1968).
'The Genetics of Bacteria and their Viruses'
Oxford. Blackwell Scientific Publications.

Heiman, C., Miller, C.G. (1978 b).
Salmonella typhimurium mutants lacking protease II.
J. Bacteriol. 135;588-594.

Heiman, C., Miller, C.G. (1978 a).
Acyl amino acid esterase mutants of Salmonella typhimurium.
Mol. Gen. Genet. 164;57-62.

Henrici, A.T. (1928).
'Morphological variation and the rate of growth of bacteria.'
Springfield, Ill. U.S.A. C.C. THOMAS.

Heppel, L.A. (1971).
The concept of periplasmic enzymes.
In 'Molecular biology: structure and functions in biological membranes'
Ed. Rothfield L.I. Academic Press, New York. pp. 224-247.

Hershko, A., Ciechanover, A. (1982).
Mechanisms of intracellular protein breakdown.
Annu. Rev. Biochem. 51;335-364.

Hershko, A., Ciechanover, A., Heller, H., Haas, A.L., Rose, I.A. (1980).
Proposed role of ATP in protein breakdown: conjugation of proteins with multiple chains of the polypeptide of ATP-dependent proteolysis.
Proc. Natl. Acad. Sci. U.S.A. 77;1783-1786.

Heussen, C. Dowdle, E.B. (1980).
Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulphate and copolymerised substrates.
Anal. Biochem. 102;196-202.

Hipkiss, A.R., Armstrong, D.W. Kushner, D.J. (1980).
Protein turnover in a moderately halophilic bacterium.
Can. J. Microbiol. 26;196-203.

Holzer, H., Betz, H., Ebner, E. (1975).
Intracellular proteinases in microorganisms.
Curr. Top. Cell Regulat. 9;103-146.

Horii, T., Ogawa, H., Nakatoni, T., Hase, T., Matsubara, H., Ogawa, H. (1981).
Regulation of SOS function: purification of E.coli lexA protein and determination of its specific site cleaved by the recA protein.
Cell 27;515-522.

Huges, D.E., Wimpenny, J.W.T. Lloyd, D. (1971).
Disintegration of microorganisms.
in 'Methods in Microbiology' 5B;30
Eds. Norris, J. Ribbons, D.W. Academic Press,New York.

Ichihara, S., Beppu, N., Muzushima, S. (1984).
Protease 1V, a cytoplasmic membrane protein of Escherichia coli, has signal peptidase activity.
J. Biol. Chem. 259;9853-9857.

Ito, J., Meinke, M., Hathaway, G., Spizizen, J. (1973).
Studies on Bacillus subtilis bacteriophage Ø15.
Virology 56;110-132.

James, G.T. (1978).
Inactivation of the protease inhibitor phenylmethylsulfonyl fluoride in buffers.
Anal. Biochem. 86;574-579.

Jinks-Robertson, S., Gourse, R.L., Nomura, M. (1983).
Expression of r-RNA and t-RNA genes in Escherichia coli: evidence for feedback regulation by products of r-RNA operons.
Cell 33;865-876.

Kaback, H.R. (1971).
Bacterial membranes.
In 'Enzyme Purification and Related Techniques' XXII;99-120.
Ed. Jacoby, W.B. Academic Press, New York.

Kari, C., Torok, I., Travers, A.A. (1977).
PpGpp cycle in Escherichia coli.
Molec. Gen. Genet. 150;249-255.

Keil-Dlouha, V., Misrahi, R., Keil, B. (1976).
The induction of collagenase and a neutral proteinase by their high MW substrates in Achromobacter iophagus.
J. Mol. Biol. 107;293-305.

Kellenberger, E., Ryter, A. & Sechaud, J. (1958).
Electron microscopic study of DNA-containing plasms. Vegetative and mature phage DNA as compared with normal nucleoids in different physiological states.
J. Biophys. Biochem. Cytol. 4;671-678.

Kemshead, J.T., Hipkis, A.R. (1974).
Degradation of abnormal proteins in Escherichia coli: relative susceptibility of canavanyl proteins and puromycin peptides to proteolysis in vitro.
Eur. J. Biochem. 45;535-540.

Kennedy, S.F., Colwell, R.R. Chapman, G.B. (1970).
Ultrastructure of a marine psychrophilic Vibrio.
Can. J. Microbiol. 16;1027-1031.

Kennedy, E.P. (1982).
Osmotic regulation and the biosynthesis of membrane-derived oligosaccharides in Escherichia coli.
Proc. Natl. Acad. Sci. U.S.A. 79;1092-1095.

Kirschner, J. Goldberg, A.L. (1981).
Nonlysosomal insulin-degrading proteinases in mammalian cells.
in 'Methods in Enzymology' 80;702-711.
Ed. Lorand, L. Academic Press, New York.

Kirsh, M., Dembinski, D.R., Hartman, P.E., Miller, C.G. (1978).
Salmonella typhimurium peptidase active on carnosine.
J. Bacteriol. 134;361-374.

Koch, R. (1884).
Uber die cholera bakterien.
Deutsche Med. Woshenschr. pp 11,8°.

Koch, A.L. (1971).
The adaptive responses of Escherichia coli to a feast and famine existence.
Adv. in Microbial. Physiol. 6;147-217.

Koch, A.L. Deppe, C.S. (1971).
In-vivo assay of protein synthesising capacity of Escherichia coli from slowly growing chemostat cultures.
J. Mol. Biol. 55;549-562.

Kowit, J.D., Goldberg, A.L. (1977).
Intermediate steps in the degradation of a specific abnormal protein in Escherichia coli.
J. Biol. Chem. 253;8350-8357.

Kowit, J.D., Choy, W.-N., Champe, S.P., Goldberg, A.L. (1976).
Role and localisation of 'Protease 1' from Escherichia coli.
J. Bacteriol. 128;776-784.

Kushner, D.J. (1978).
Life in high salt and solute concentrations: halophilic bacteria.
Bacteriol. Rev. 38;272-290.

Lagosky, P.A. Chang, F.N. (1980).
Influence of amino acid starvation on guanosine 5'-diphosphate, 3'-diphosphate basal level synthesis in Escherichia coli.
J. Bacteriol. 144;499-508.

Lagosky, P.A. Chang, F.N. (1978).
The extraction of guanosine 5'-diphosphate, 3'-diphosphate (ppGpp) from Escherichia coli using low pH reagents; a reevaluation.
Biochem. Biophys. Res. Commun. 84; 1016-1024.

Lamond, A.I. (1985).
The control of stable RNA synthesis in bacteria.
Trends in Biochem. Sci. 10;271-274.

Lamond, A.I.,Travers, A.A. (1985).
Stringent control of bacterial transcription.
Cell 41;6-8.

Larrabee, K.L., Phillips, J.O., Williams, G.J., Larrabee, A.R. (1980).
The relative rates of protein synthesis and degradation in a growing culture of Escherichia coli.
J. Biol. Chem. 255;4125-4130.

Lau, C., Richards, F.M. (1976).
Behavior of colicins E1, E2, and E3 attached to Sephadex beads.
Biochemistry 15;666-671.

Lewin, B.M. (1977).
In 'Gene Expression. III'
New York. J. Wiley & Sons.

Lingappa, V.R., Devillers-Thiery, A., Blobel, G. (1977).
Nascent prehormones are intermediates in the biosynthesis of authentic bovine pituitary growth hormone and prolactin.
Proc. Natl. Acad. Sci. U.S.A. 74;2432-2436.

Little, J.W., Mount, D.W. (1982).
The SOS regulatory system of Escherichia coli.
Cell 29;11-22.

Little, R., Ryals, J., Bremer, H. (1983).
Physiological characterisation of Escherichia coli rpoB mutants with abnormal control of ribosome synthesis.
J. Bacteriol. 155;1162-1170.

Liu, C.-J., Chopra, A. K., Strnadova, M. Chaloupka, J. (1984).
Degradation of abnormal proteins in growing yeast.
F.E.M.S. Microbiol. Lett. 21;313-317.

Lowry, O.H., Rosebrough, N.J. Farr, A.L. Randall, R.J. (1951).
Protein measurement with the Folin phenol reagent.
J. Biol. Chem. 254;8194-8200.

Lund, E. Kjeldgaard, N.O. (1972).
Metabloism of guansoine tetraphosphate in Escherichia coli.
Eur. J. Biochem. 28;316-326.

Maaloe, O. (1979).
In 'Biological Regulation and Development'
Ed. Goldberger, R.F. 1;487-542.
Plenum, New York.

Maaloe, O., Kjeldgaard, N.O. (1966).
'Control of macromolecular synthesis.'
Benjamin, New York.

MacGregor, C.H., Bishop, C.W., Blech, J.E. (1979).
Localisation of the proteolytic activity in the outer membrane of Escherichia coli.
J. Bacteriol. 137;574-583.

Maeda, A., Nomura, M. (1966).
Interaction of colicins with bacterial cells. 1. Studies with radioactive colicins.
J. Bacteriol. 91;685-694.

Mandelstam, J. (1958).
The free amino acids in growing and non-growing populations of Escherichia coli.
Biochem. J. 69;103-110.

Mandelstam, J. (1960).
The intracellular turnover of protein and nucleic acids and its role in biochemical differentiation
Bacteriol. Rev. 24;289-308.

Mandelstam, J. (1957).
Turnover of proteins in starved bacteria and its relationship to the induced synthesis of enzymes.
Nature 179;1179-1181.

Mandlestam, J., Waites, W.M. (1968).
Sporulation in Bacillus subtilis: the role of exoprotease.
Biochem. J. 109;793-801.

Markovitz, A. (1977).
Genetics and regulation of bacterial capsular polysaccharide biosynthesis and radiation sensitivity.
In 'Surface Carbohydrates of the Procaryotic Cell.' Ed. Sutherland, I. 415-462 Academic press, London.

Markwell, M.A.K., Haas, S.M., Bieber, L.L. Tolbert, N.C. (1978).
A modification of Lowry procedure to simplify protein determinations in membrane and lipoprotein samples.
Anal. Biochem. 87;206-210.

Marshall, K.C. (1985).
Bacterial adhesion in oligotrophic habitats.
Microbiol. Sci. 2;321-326.

Maurizi, M.R., Brabson, J.S., Switzer, J.S. (1978).
Immunochemical studies of the inactivation of aspartate transcarbamylase by stationary phase Bacillus subtilis cells.
J. Biol. Chem. 253;5585-5593.

McHugh, G.L., Miller, C.G. (1974).
Isolation and characterisation of proline peptidase mutants of Salmonella typhimurium.
J. Bacteriol. 120;364-371.

Meyer, E., Switzer, R.L. (1979).
Regulation of Bacillus subtilis glutamine phosphoribosylpyrophosphate amido-transferase activity by end products.
J. Biol. Chem. 254;5397-5402.

Miller, C.G., Green, L. (1981).
Degradation of abnormal proteins in peptidase-deficient mutants of Salmonella typhimurium.
J. Bacteriol. 147;925-930.

Miller, C.G., Mackinnon, K. (1974).
Peptidase mutants of Salmonella typhimurium.
J. Bacteriol. 120;355-363.

Miller, C.G., Schwartz, G. (1978).
Peptidase-deficient mutants of Escherichia coli.
J. Bacteriol. 135;603-611.

Miller, C.G., Zipser, D. (1977).
Degradation of Escherichia coli beta-galactosidase fragments in protease-deficient mutants of Salmonella typhimurium.
J. Bacteriol. 130;347-353.

Milstein, C., Brownlee, G.G., Harrison,T.M., Mathews, M.B. (1972).
A possible precursor of immunoglobulin light chains
Nature New Biol. 239;117-120.

Mizusawa, S., Gottesman, S. (1983).
Protein degradation in Escherichia coli: the lon gene controls the stability of Sul A protein.
Proc. Natl. Acad. Sci. U.S.A. 80;358-362.

Moore, G. Crichton, R.R. (1973). Reductive methylation: a method for prepating functionally active radioactive ribosomes.
F.E.B.S. Lett. 37;74-78.

Morita, R.Y. (1982).
Starvation-survival of heterotrophs in the marine environment.
Adv. Microbial Ecology 6;171-198.

Morona, R., Reeves, P. (1984).
Detection of several diisopropylfluorophosphate-binding proteins in the outer membrane of Escherichia coli K12.
F.E.M.S. Microbiol. Letts. 23;179-182.

Morrison, S.L., Zipser, D. (1970).
Polypeptide products of nonsense mutations. I. Termination fragments from nonsense mutations in the Z gene of the Lac operon of Escherichia coli
J. Mol. Biol. 50;359-371.

Mosteller, R.D., Nishimoto, K.R., Goldstein, R.V. (1977).
Inactivation and partial degradation of phosphoribosyl-anthranilate isomerase - indoleglycerol phosphate synthetase in nongrowing cultures of Escherichia coli.
J. Bacteriol. 131;153-162.

Mosteller, R.D., Goldstein, R.V., Nishimoto, K.R. (1980).
Metabolism of individual proteins in exponentially growing Escherichia coli.
J. Biol. Chem. 255;2524-2532.

Munoz, L., Sadaie, Y., Doi., R.H. (1978).
Spore coat protein of Bacillus subtilis: structure and precursor synthesis.
J. Biol. Chem. 253;6694-6701.

Murakami, K., Voellmy, R., Goldberg, A.L. (1979).
Protein degradation is stimulated by ATP in extracts of Escherichia coli. J. Biol. Chem. 254;8194-8200.

Murakami, K., Voellmy, R. Goldberg, A.L. (1979).
Protein degradation is stimulated by ATP in extracts of Escherichia coli.
J. Biol. Chem. 254;8194-8200.

Nakayama, T, Munoz, L.E., Sadaie, Y., Doi, R.M. (1978).
Spore coat synthesis in cell-free systems from sporulating cells of Bacillus subtilis.
J. Bacteriol. 135;952-960.

Nath, K., Koch., A.L. (1970).
Protein degradation in Escherichia coli. 1. Measurement of rapidly and slowly decaying components.
J. Biol. Chem. 245;2889-2900.

Nath, K., Kock, A.L. (1971).
Protein degradation in Escherichia coli. 11. Strain differences in the degradation of protein and nucleic acid.
J. Biol. Chem. 246;6956-6967.

Ninio, J. (1975).
Kinetic amplification of enzyme discrimination.
Biochimie 57;587-595.

Nomura, M.J., Sidikaro, K., Jakes, K., Zinder, N. (1974).
Effects of colicin E3 on bacterial ribosomes.
In "Ribosomes" Eds. Nomura, M., Tissiers, A. Lengyel, P. 805-813.
Cold Spring Harbor Laboratory, Cold Spring Harbor,N.Y.

Nomura, M., Gourse, R., Baughman, G. (1985).
Regulation of the synthesis of ribosomes and ribosomal components.
Annu. Rev. Biochem. 53;75-117.

Nomura, M. (1967).
Colicins and related bacteriocins.
Annu. Rev. Microbiol. 21;257-284.

Nossal, N.G. Hepple, A. (1966).
The release of enzymes by osmotic shock from Escherichia coli in exponential phase.
J. Biol. Chem. 241;3055-3062.

Novitsky, J.A., Morita, R.Y. (1976).
Morphological characterisation of small cells resulting from nutrient starvation of a psychrophilic marine Vibrio.
J. Bacteriol. 32;617-622.

Novitsky, J.A., Morita, R.Y. (1977).
Survival of a psychrophilic marine Vibrio under long-term nutrient starvation.
App. Env. Microbiol. 33;635-641.

Novitsky, J.A., Morita, R.Y. (1978 a).
Possible strategy for the survival of marine bacteria under starvation conditions.
Marine Biology 48;289-295.

Novitsky, J.A., Morita, R.Y. (1978 b).
Starvation-induced barotolerence as a survival mechanism of a psychrophilic marine Vibrio in the waters of the Antarctic convergence.
Marine Biology 49;7-10.

O'Farrell, P.M. (1978).
The suppression of defective translation by ppGpp and its role in the stringent response.
Cell 14;545-547.

Olden, K., Goldberg, A.L. (1978).
Studies on the energy requirement for intracellular protein degradation in Escherichia coli.
Biochim. et Biophys. Acta 542;385-398.

Oliver, C.N. Stadtman, E.R. (1983).
A proteolytic artifact associated with the lysis of bacteria by egg white lysozyme.
Proc. Natl. Acad. Sci. U.S.A. 80;2156-2160.

Osborn, M.J. Munson, R. (1974).
Separation of the inner (cytoplasmic) and outer membranes of Gram-negative bacteria.
In 'Methods in Enzymology.' 31;642-653. Academic Press, New York.

Pacaud, M. (1976).
Purification of protease II from Escherichia coli by affinity chromatography and septation of two enzyme species from cells harvested at late log phase.
Eur. J. Biochem. 64;199-204.

Pacaud, M., Richaud, C. (1975).
Protease II from Escherichia coli: purification and characterisation.
J. Biol. Chem. 250;7771-7779.

Pacaud, M., Sibilli, L., LeBras, G. (1976).
Protease 1 from Escherichia coli: some physicochemical properties and substrate specificity.
Eur. J. Biochem. 69;141-151.

Pacaud, M. (1982).
Identification and localisation of two membrane-bound esterases from Escherichia coli.
J. Bacteriol. 149;6-14.

Phizicky, E.M., Roberts, J.W. (1981).
Induction of SOS functions; regulation of proteolytic activity of E.coli Rec A protein by interaction with DNA and nucleoside triphosphate.
Cell 25;259-267.

Phizicky, E.M., Roberts, J.W. (1980).
Kinetics of Rec A protein directed inactivation of repressors of phage lambda and phage P22.
J. Mol. Biol. 139;319-328.

Pine, M.J. (1965).
Heterogeneity of protein turnover in Escherichia coli.
Biochim. et Biophys. Acta 104;439-456.

Pine, M.J. (1966).
Metabolic control of intracellular proteolysis in growing and resting cells of Escherichia coli.
J. Bacteriol. 92;847-850.

Pine, M.J. (1970).
Steady state measurement of the turnover of amino acids in the cellular proteins of growing Escherichia coli: existence of two kinetically distinct reactions.
J. Bacteriol. 103;207-215.

Pine, M.J. (1972).
Turnover of intracellular proteins.
Annu. Rev. Microbiol. 26;103-126.

Pine, M.J. (1973).
Regulation of intracellular proteolysis in Escherichia coli.
J. Bacteriol. 115;107-116.

Platt, T., Miller, J.M., Weber, K. (1970).
In Vivo degradation of Mutant lac Repressor.
Nature 228;1154-1156.

Potier, P., Hipkiss, A.R. Kushner, D.J. (1985).
Protein turnover in a psychrotrophic bacterium.
Arch. Microbiol. 142;28-33.

Prouty, W.F., Goldberg, A.L. (1972).
Fate of abnormal proteins in E.coli. Accumulation in intracellular granules before catabolism.
Nature New Biol. 240;147-150.

Prouty, W.F., Karnovsky, M.J., Goldberg, A.L. (1975).
Degradation of abnormal proteins in Escherichia coli. Formation of protein inclusions in cells exposed to amino acid analogues.
J. Biol. Chem. 250;1112-1122.

Radding, C.M. (1981).
Recombination activities of E.coli Rec A protein.
Cell 25;3-4.

Ramagopal, S. (1984).
Metabolic changes in ribosomes of Escherichia coli during prolonged culture in different media.
Eur. J. Biochem. 140;353-361.

Reeve, C.A., Brockman, A.T., Matin, A. (1984).
Role of protein degradation in the survival of carbon-starved Escherichia coli and Salmonella typhimurium.
J. Bacteriol. 157;758-763.

Reid, G.C., Woods, D.R., Robb, F.T. (1980).
Peptone induction and rifampicin insensitive collagenase production by Vibrio alginolyiticus.
J. Bacteriol. 142;447-454.

Rhaese, H.J., Hoch, J.A., Groscurth, R. (1977).
Studies on the control of development: isolation of Bacillus subtilis mutants blocked early in sporulation and defective in synthesis of highly phosphorylated nucleotides.
Proc. Natl. Acad. Sci. U.S.A. 74;1125-1129.

Rhaese, H.J., Grade, R., Dichtelmuller, H. (1976).
Studies on the control of development. Correlation of initiation of differentiation with synthesis of highly phosphorylated nucleotides in Bacillus subtilis.
Eur. J. Biochem. 64;205-213.

Rhaese, H.J., Groscurth, R. (1978).
Studies on the control of development: synthesis of two highly phosphorylated nucleotides depends on changes in the composition of ribosomes at the beginning of sporulation in Bacillus subtilis.
Eur. J. Biochem. 85;517-528.

Rice, R. H. Means, G.E. (1971).
Radioactive labelling of proteins in vitro.
J. Biol. Chem. 246;831-832.

Robb, F.T., Robb, S.M., Mothibeli, M.A., Woods, D.R. (1982).
Leucine uptake and bacteriophage adsorption in a Vibrio strain.
South African J. Sci. 78;122-123.

Robb, S.M. (1979).
Bacteriophage growth on stationary phase Achromobacter strains.
Ph.D thesis, Rhodes University, Grahamstown.

Robb, S.M., Robb, F.T., Woods,D.R. (1980).
Physiological and morphological characteristics of stationary phase Vibrio cells able to support phage growth.
J. Gen. Microbiol. 119;405-412.

Robb, S.M., Woods, D.R., Robb, F.T., Struthers, J.K. (1977).
Rifampicin-resistant mutant supporting bacteriophage growth on stationary phase Achromobacter cells.
J. Gen. Virol. 35;117-123.

Robb, S.M., Woods, D.R., Robb, F.T. (1978).
Phage growth characteristics on stationary phase Achromobacter cells.
J. Gen. Virol. 41;265-272.

Roberts, J.W., Roberts, C.W., Craig, N.L. (1978).
Escherichia coli recA gene product inactivates phage lambda repressor.
Proc. Natl. Acad. Sci. U.S.A. 75;4714-4718.

Rotman, B., Spiegelman, S. (1954).
On the origin of the carbon in the induced synthesis beta-galactosidase in Escherichia coli.
J. Bacteriol. 68;419-429.

Ruppen, M.E., Garner, G., Sadoff, H.L. (1983).
Protein turnover in Azotobacter vinelandii during encystment and germination.
J. Bacteriol. 156;1243-1248.

Russell, N.J. Kogut, M. (1985).
Haloadaptation: salt sensing and cell-envelope changes.
Microbiol. Sci. 2;345-350.

Ryals, J., Little, R., Bremer, H. (1982 a).
Control of rRNA and tRNA syntheses in Escherichia coli by guanosine tetraphosphate.
J. Bacteriol. 151;1261-1268.

Ryals, J., Little, R., Bremer, H. (1982 b).
Control of RNA synthesis in Escherichia coli after a shift to higher temperature.
J. Bacteriol. 151;1425-1432.

Sadoff, H.L. (1975).
Encystment and germination in Azotobacter vinelandii.
Bacteriol. Rev. 39;516-539.

Sadoff, H.L., Berke, E., Loperfido, B. (1971).
Physiological studies of encystment in Azotobacter vinelandii.
J. Bacteriol. 105;185-189.

Sands, M.K., Roberts, R.B. (1952).
The effects of a tryptophan-histidine deficiency in a mutant of Escherichia coli.
J. Bacteriol. 63;505-511.

Schachtele, C.F., Anderson, D.L., Rogers, P. (1968).
Mechanism of canavanine death in Escherichia coli. II. Membrane-bound canavanyl-protein and nuclear disruption.
J. Mol. Biol. 33;861-872.

Schaller, K., Nomura, M. (1976).
Colicin E2 is a DNA endonuclease.
Proc. Natl. Acad. Sci. U.S.A. 73;3989-3995.

Schimke, R.T. (1973).
Control of enzyme levels in mammalian tissues.
Adv. Enzymol. 37;135-187.

Setlow, P. (1978 a).
Purification and characterisation of additional low molecular-weight basic proteins degraded during germination of Bacillus megaterium spores.
J. Bacteriol. 136;331-340.

Setlow, P. (1978 b).
Degradation of dormant spore protein during germination of Bacillus megaterium spores.
In "Limited Proteolysis in Microorganisms" Eds. Cohen G.N., Holzer H. Berthesda Md: DHEW

Shineberg, B., Zipser, D. (1973).
The lon gene and degradation of beta-galactosidase nonsense fragments.
J. Bacteriol. 116;1469-1471.

Silverman, R.H., Atherly, A.G. (1979).
The search for guanosine tetraphosphate (ppGpp) and other unusual nucleotides in eukaryotes.
Microbiol. Rev. 43;27-41.

Sonnenshein, A.L., Roscoe, D.H. (1969).
The course of Oe infection in sporulating cells of Bacillus subtilis strain 3610.
Virology 39;265-276.

Spadaro, A., Spena, A., Santonastasa, V., Donini, P. (1981).
Stringency without ppGpp accumulation.
Nature 291;256-258.

Speiser, S., Etlinger, J.D. (1982).
Loss of ATP-dependent proteolysis with maturation of reticulocytes and erythrocytes.
J. Biol. Chem. 257;1422-1427.

Speiser, S., Etlinger, J.D. (1983).
ATP stimulates proteolysis in reticulocyte extracts by repressing an endogenous protease inhibitor.
Proc. Natl. Acad. Sci. U.S.A. 80;3577-3580.

Spudich, J.A., Kornberg, A. (1968).
Biochemical studies of bacterial sporulation and germination VII. Protein turnover during sporulation of Bacillus subtilis.
J. Biol. Chem. 243;4600-4605.

St John, A.C., Conklin, K., Rosenthal, E., Goldberg, A.L. (1978).
Further evidence for the involvement of charged t-RNA and guanosine tetraphosphate in the control of protein degradation in Escherichia coli. J. Biol. Chem. 253;3945-3951.

St John, A.C., Goldberg, A.L. (1980).
Effects of starvation for potassium and other inorganic ions on protein degradation and ribonucleic acid synthesis in Escherichia coli.
J. Bacteriol. 143;1223-1233.

St. John, A.C., Goldberg, A.L. (1978).
Effects of reduced energy production on protein degradation, guanosine tetraphosphate, and RNA synthesis in Escherichia coli.
J. Biol. Chem. 253;2705-2711.

Stent, G.S., Brenner, S. (1961).
A genetic locus for the regulation of ribonucleic acid synthesis.
Proc. Natl. Acad. Sci. U.S.A. 47;2005-2014.

Stokes, J.L., Parson, W.L. (1968).
Role of poly-beta-hydroxybutyrate in survival of Sphaerotilus discophorus during starvation.
Canadian J. Microbiol. 14;785-789.

Strange, R.E., Dark., F.A., Ness, A.G. (1961).
The survival of stationary phase cells of Aerobacter aerogenes stored in aqueous suspensions.
J. Gen. Microbiol. 25;61-76.

Strongin, A.Ya., Gorodetsky, D.I., Stepanov, V.M. (1979).
The study of Escherichia coli proteases, intracellular serine protease of E.coli - an analogue of bacillus proteases.
J. Gen. Microbiol. 110;443-451.

Strongin, A.Ya., Izotova, L.S., Abramov, Z.T., Gorodetsky, D.I., Ermakova, L.M., Baratova, L.A., Belyanova, L.P., Stepanov, V.M. 1978).
Intracellular serine protease of Bacillus subtilis: sequence homology with extracellular subtilisins.
J. Bacteriol. 133;1401-1411.

Sussman, A.J., Gilvarg, C. (1969).
Protein turnover in amino acid starved strains of Escherichia coli K12 differing in their ribonucleic acid control.
J. Biol. Chem. 244;6304-6308.

Swamy, K.H.S. Goldberg, A.L. (1982).
Subcellular distribution of various proteases in Escherichia coli.
J. Bacteriol. 149;1027-1033.

Swamy, K.H.S., Goldberg, A.L. (1981).
E.coli contains eight soluble proteolytic activities, one being ATP-dependent.
Nature 292;652-654.

Swamy, K.H.S., Goldberg, A.L. (1982).
Subcellular distribution of various proteases in Escherichia coli.
J. Bacteriol. 149;1027-1033.

Sy, J., Ogawa, V., Lipmann, F. (1973).
Non-ribosomal synthesis of guanosine 5',3' polyphosphates by the ribosomal wash of stringent Escherichia coli.
Proc. Natl. Acad. Sci. U.S.A. 70;2145-2148.

Thomas, T.D., Batt, R.D. (1968).
Survival of Streptococcus lactis in starvation conditions.
J. Gen. Microbiol. 50;367-382.

Thomson, J.A., Woods, D.R., Welton, R.L. (1972).
Collagenolytic activity of aerobic halophiles from hides.
J. Gen. Microbiol. 70;315-319.

Thomson, J.A., Woods, D.R. (1974).
Bacteriophages and cryptic lysogeny in Achromobacter.
J. Gen. Virol. 22;153-157.

Thomson, J.A., Woods, D.R. (1973).
Properties of spheroplasts of a halotolerant Achromobacter strain and their infection with bacteriophage deoxyribonucleic acid.
J. Gen. Microbiol. 74;71-76.

Thomson, J.T. (1973).
Genetic studies on collagenolytic Achromobacter strains and their bacteriophages.
Ph.D thesis, Rhodes University, Grahamstown.

Tokunaga, M., Loranger, J.M., Wolfe, P.B., Wu, H.C. (1982).
Prolipoprotein signal peptidase in Escherichia coli is distinct from the M13 procoat protein signal peptidase.
J. Biol. Chem. 257;9922-9925.

Urba, R.C. (1959).
Protein breakdown in Bacillus cereus.
Biochem. J. 71;513-518.

Van Verseveld, H.W., Arbige, M., Chesbro, W.R. (1984).
Continuous culture of bacteria with biomass retention.
Trends in Biotechnol. 2;8-12.

Van Verseveld, H.W., Braster, M., Chesbro, W.R., Stouthamer, A.H. (1984). Very slow growth of Paracoccus denitrificans in a recycling fermenter. Arch. Microbiol. 137;176-183.

Voellmy, R.W., Goldberg, A.L. (1980).
Guanosine-5'-diphosphate-3'-diphosphate (ppGpp) and the regulation of protein breakdown in Escherichia coli.
J. Biol. Chem. 255;1008-1014.

Voellmy, R.W., Goldberg, A.L. (1981).
ATP-stimulated endoprotease is associated with the cell membrane of E.coli.
Nature 290;419-421.

Vogel, A.I. (1961).
The Mohr titration.
In 'A Textbook of Quantitative Inorganic Analysis.' p. 259
Longman, London.

Watson, D.H., Sherratt, D.J. (1979).
In vivo proteolytic cleavage of colicins requires specific receptor binding.
Nature 278;362-364.

Waxman, L., Fagan, J.M., Tanaka, K., Goldberg, A.L. (1985).
A soluble ATP-dependent system for protein degradation from murein erythroleukemia cells. Evidence for a protease which requires ATP hydrolysis but not ubiquitin.
J. Biol. Chem. 260;11994-12000.

Weiss, W.J., Luria, S.E. (1978).
Reduction of membrane potential, an immediate effect of colicin K.
Proc. Natl. Acad. Sci. U.S.A. 75;2483-2487.

Welton, R.L., Woods, D.R. (1974).
Collagenase production by Achromobacter iophagus.
Biochim. et Biophys. Acta 384;228-234.

Welton, R.L., Woods, D.R. (1973).
Halotolerant collagenolytic activity of Achromobacter iophagus.
J. Gen. Microbiol. 75;191-196.

Wheatley, D.N., Giddings, M.R., Inglis, M.S., Robertson, J.H. (1978).
In vivo degradation of analogue proteins by suspension cultured Hela cells.
Microbiol. Lett. 4;233-245.

Wickner, W., Brutlag, D., Schekman, R. Kornberg, A. (1972).
RNA synthesis initiates in vitro conversion of M13 DNA to its replicative form. Proc. Natl. Acad. Sci. U.S.A. 69;965-969.

Wickner, W. (1979).
The assembly of proteins into biological membranes: the membrane trigger hypothesis.
Annu. Rev. Biochem. 48;23-45.

Wiebe, W.J. Chapman, G.B. (1968).
Fine structure of selected marine pseudomonads and achromobacters.
J. Bacteriol. 95;1862-1873.

Willetts, N.S. (1965).
Protein degradation during diauxic growth of Escherichia coli.
Biochim. Biophys. Res. Commun. 20;692-696.

Willetts, N.S. (1967).
Intracellular protein breakdown in growing cells of Escherichia coli.
Biochem. J. 103;462-466.

Witholt, B., Boekhout, M., Brock, M., Krigma, J., van Heerikhuizen, H. de Leij, L. (1976).
An efficient and reproducible procedure for the formation of spheroplasts from variously grown Escherichia coli.
Anal. Biochem. 74;160-170.

Woods, D.R. (1976).
Bacteriophage growth on stationary phase Achromobacter cells.
J. Gen. Virol. 32;45-50.

Woods, D.R., Thomson, J.A. (1975).
Unstable generalised transduction in Achromobacter.
J. Gen. Microbiol. 88;86-92.

Woods, D.R., Welton, R.L., Thomson, J.A., Cooper, D.R. (1972).
The microbiology of curing and tanning processes, part IV. Collagenolytic activity of bacteria from raw and cured hides.
J. American Leather Chemists' Assoc. 67;217-225.

Yehle, C.O., Doi, R.H. (1967).
Differential expression of bacteriophage genomes in vegetative and sporulating cells of Bacillus subtilis.
J. Virol. 1;935-947.

Yen, C., Green, L., Miller, C.G. (1980).
Peptide accumulation during growth of peptidase deficient mutants.
J. Mol. Biol. 143;35-48.

Yonetani, T. (1967).
Studies on cytochrome C peroxidase X. Crystalline apo- and reconstituted haloenzymes.
J. Biol. Chem. 242;5008-5013.

Zwisinski, C., Date, T., Wickner, W. (1981).
Leader peptidase is found in both the inner and outer membranes of Escherichia coli.
J. Biol. Chem. 256;3593-3597.

U N I V E R S I T Y O F C A P E T O W N

M E M O R A N D U M

FROM: Nuala Magnus
DDB Office

TO: Sarah Daitsch
Jagger Library

4 February 1988

I have pleasure in advising that the following theses of December 1987 graduands are ready for collection:

A L Bosman,
R I Bust,
K G Clarke,
G Dennill
N G Car
L A F Casciola
M R W Ehlers
P A Faber
D L Hawla
J B Kruskal
M R Levin
W M Mabusela
M Purves
A L Reysenbach
A Pouris
G N Nurick
D M M McClean
M Todes NB This candidate submitted his corrected copies BOUND, he is going to the USA soon to do some work there but can be contacted if you would prefer unbound copies.
D Szeinfeld Loef
G Wallis
F G Waters

8 FEB 1988